

FIELD APPLICATION FOR EXPERIMENTAL INACTIVATED MULTIVALENT *P. multocida* AND AVIAN INFLUENZA (H9N2) VACCINE IN POULTRY

Selim S. Salama^{1*}, Heba A. Abdelhady², Lamiaa Atia³

¹Central Laboratory for Evaluation of veterinary Biologics (CLEVB), Cairo, Egypt, ²Animal Health Research Institute (AHRI), Alexandria branch, Alexandria, Egypt, ³Animal Health Research Institute (AHRI), Banha branch, Qalyubia, Egypt

*Corresponding author, E-mail: selimsalama2000@yahoo.com

Abstract: Fowl cholera (FC) and Avian Influenza (AI) are two of the major economically important respiratory and septicaemic disease of poultry in Egypt and allover the world. A field trails was conducted to evaluate the immunogenicity of an experimentally multivalent inactivated Fowl cholera (*P. multocida*) and Avian Influenza (H9N2) vaccine. The In this study a combined inactivated Montanide ISA fowl cholera and AI vaccine was prepared then the immunization potency and protective efficacy were evaluated through experimental application on different poultry breeds including broiler breeder, broiler and turkey flocks. The peak of humeral immune response against *P. multocida* as measured by ELISA was at the 5th week post vaccination and at 3rd week post boosting all over the used poultry flocks. At the same time, AI immune response as measured by HI reached the peak at the 6th week post vaccination and at 2nd week post boosting. Regarding the challenge test, the protection levels were 76.6, 70 and 80% and 90, 86.6 and 93.3% against *P. multocida* while protection levels were 83.3, 80 and 86.6% and 93.3, 90 and 96.6% against AI virus after challenge with the used virulent strains post single and booster dose respectively. In conclusion the combined prepared fowl cholera and AI vaccine succeeded in eliciting protective antibody titres and full protection against both fowl cholera and AI diseases.

Key words: *Pasteurella multocida*; AI; vaccine; ELISA; PHA; HI; challenge test

Introduction

Fowl cholera (1) and AI (2) are two of the major economically important respiratory and septicaemic diseases of poultry in Egypt and allover the world. They are highly contagious diseases causing devastating economic losses to the poultry industry through death, weight loss and condemnation of carcasses worldwide.

Fowl Cholera caused by *P. multocida* which belongs to the family *Pasteurellaceae* and classified into five groups based on capsular antigens and into 16 serotypes based on LPS antigens (3). It occurs sporadically or enzootically as peracute, acute or chronic form all over the world (4). Mortality may range from only few percent to nearly 100% and recovered birds may remain as carriers even after 9 weeks after infection (1).

Avian influenza (AI) is an infectious respiratory disease of birds caused by avian influenza type A viruses that are members of the family Orthomyxoviridae (5). The H9N2 avian influenza virus (AIV) was reported to be of low pathogenicity in chickens (6, 7) causing minimal clinical signs other than a slight drop in egg production but greatly has immunosuppressive effect (8) confirmed that the control of H9N2 viruses in poultry is important.

Combination of bacterial and viral vaccines which contain multiple antigens has many benefits for the manufacturer as it reduce production costs, for the administrator as it save time, effort and simplify the immunization schedule and for the animals as it minimize stress of multiple vaccinations (9).

The aim of this study is to evaluate the usage of a combined vaccine against FC and AI diseases prepared with Montanide ISA 206 as adjuvant under field condition.

Materials and methods

Strains used

a- Pasteurella multocida serotypes (1, 3, and 4): obtained from the Strain Bank, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Cairo, Egypt.

b- AI H9N2: isolated in the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), yearling 2015, Cairo, Egypt.

Vaccine Preparation

a- Preparation of inactivated Pasteurella multocida antigenic phase

A virulent local strains of *P. multocida* Serotypes 1, 3, 4 and D2 were propagated in brain heart infusion broth (BHI) at 37 °C for 24 hours to obtain a dense culture containing approximately 2×10^9 CFU of each strain. After inactivation by addition of 0.1% formalin, each culture was tested for purity, safety and sterility as mentioned by (10). Finally, cultures were equally mixed together then preserved with 0.01% of thiomersal and stored at 4 C° until use.

b- Preparation of inactivated AI antigenic phase

Propagation and titration of AI H9N2 that locally isolated were done in SPF 9 -10 day old ECE according to (11) and (12). Its titer was 10^9 EID₅₀/ml. Inactivation of AI virus was carried out using binary ethelenamine 0.1M with final concentration 0.01M. Inactivation and testing of inactivation was done according to (13).

c- Preparation of combined inactivated FC and AI vaccine by using ISA 206

A combined vaccine of *P. multocida* and AI was prepared water in oil in water emulsion by mixing equal volumes of the inactivated *P. multocida* antigenic phase and inactivated AI antigenic phase to form an aqueous phase and immersed in equal volume of Montanide ISA 206 oil adjuvant (SEPPIC, cosmetics, pharmacy division, Paris, France).

Quality control of the prepared vaccine

The vaccine was tested for sterility and safety following the standard international protocol as described by OIE (14) before the usage in the field trial.

Experimental design

A separate broiler breeder, broiler and turkey flock houses were used through the experimental work of this study. Birds were vaccinated I/M at the age of six weeks, 2 weeks and 4 weeks respectively with 0.5ml / bird with the prepared combined vaccine. Random 60 birds from each group were transferred to a specific isolator to be challenged 3 weeks post first vaccination. A group of the rest birds of the different flocks were then received booster dose and 3 weeks apart challenge test were done. Also a group of the broiler were kept to continue in a special house to complete the period of experiment. A group of bird per each flock were kept as non-vaccinated control group. Random serum samples were collected from all flocks all over the experimentation period.

Enzyme Linked Immunosorbent Assay (ELISA) ELISA was performed according to the manufacturer instruction (ID-VET) for determining *P. multocida* antibody titer.

Haemagglutination inhibition (HI) test

It was carried out according to OIE (14) for the evaluation of immune response against AI.

Challenge test

a- Challenge with virulent P. multocida strains.

This test was done according to (15). Vaccinated birds (30 per each flock) as well as non-vaccinated (15 per each flock) were challenged by inoculation of 0.1ml of 2×10^2 CFU of *P. multocida* Serotypes 1, 3, and 4 (10 vaccinated and 5 non vaccinated birds for each serotype) via intramuscular route 3 weeks after both first and booster dose vaccination. All birds were observed for 14 days and mortalities were recorded.

b- Challenge with AI H9N2 strain

This was done according to (16). 30 vaccinated as well as 10 non-vaccinated birds per each flock were challenged by inoculation of 0.1ml of AI 10^6 EID₅₀ strain via intranasal route 3 weeks after first and booster dose of vaccination then cloacal swaps were collected on 1, 3 and 5 days post challenge to check virus shedding and protection obtained.

Results and discussion

Control of fowl cholera and avian influenza are still subject of interest and usually attract the attention of researchers to know more about diseases, epidemiology and how to control in susceptible birds. Despite the prophylactic measures for protection against both diseases using various vaccines and different vaccination programmes, mortality and losses still occur due to respiratory diseases. Combination of bacterial and viral vaccines has many benefits for the manufacturer as it reduce production costs, save time, effort and simplify the immunization schedule. Also it minimize stress of multiple vaccinations on the animals. Vaccination is still considered one of the major tools for controlling both of the two diseases. Vaccine efficacy depend on many variables, such as the nature, the amount of antigen administrated and the presence of adjuvant to enhance immunogenicity (17).

FC and AI vaccine was prepared using Montanide ISA 206 and evaluated by measuring humeral immune response and vaccination challenge assays against both in this field study.

Assessment of Quality control measures for the prepared inactivated vaccine indicated that, the prepared vaccine was completely sterile, haven't any bacterial or fungal contaminants when tested on specific bacteriologic and fungal media. In addition there was no local, systemic reaction or mortalities were recorded in inoculated chicks, and these denote to the safety of the prepared vaccine.

Regarding the potency of the prepared vaccine, the serological tests carried out on serum samples obtained from vaccinated chickens with the combined FC and AI vaccine revealed that such birds exhibited detectable *P. multocida* antibodies from the first week post vaccination as shown in Table (1). The ELISA antibody titers increased gradually till reach its maximum level at the 5th week post first vaccination where it records 1247, 1197 and 1226 in case of broiler breeder, broiler and turkey flocks respectively. This is consistent with (18) who reported that the antibody titres significantly increased 3 weeks after primary vaccination. Booster doses caused a rapid shooting in the antibody titer and reached its highest level at the end of 3rd week post boosting. The highest antibody titers were 1832, 1578 and 1801, respectively.

It is clear that all birds showed a secondary response greater than that seen in birds vaccinated once, these findings was in accordance with that recorded by (15) who stated that a significant immunological stimulus had been elicited by the 2nd exposure and (19) who reported that the immune response of secondary immunization was significantly higher than the primary immunization. On the other hand (20) evaluated the immune response induced by several adjuvants included in *Pasteurella multocida* vaccines and stated that there is no significant decrease of antibody titres was observed between 4 and 8 weeks after vaccination.

The inoculation of booster dose of combined vaccine at the start of 4th week caused a rise of antibodies titer where reached the peak against

P. multocida at the 3rd week post boosting. From these investigations, it was clear that for longer term protection against FC, the vaccines should be given at two doses, at least three weeks apart.

As regards to the HI test that carried out on serum samples obtained from vaccinated chickens with the prepared combined FC and AI vaccine, it revealed that there was an increase in AI antibodies with titer of 6.4, 5.8 and 6.4 log₂ when measured at the 3rd week post single dose of vaccination which raised gradually up to its maximum level at the 5th week recording 7.8, 7.2 and 7.6 in broiler breeder, broiler chickens and turkey respectively as shown in Table (2). Meanwhile the inoculation of booster dose after 3wks caused a rapid rise of antibodies titer where the peak of AI antibody titer was 8.6, 8.0 and 8.2 log₂ by the 3rd week post boosting respectively. The same results obtained by (21) who determined the efficacy of two oil emulsion (ISA 206, ISA70) HA subunit vaccine derived from H5N1 virus.

The same results obtained by (22) who evaluated the quality of oil emulsion AI H9N2 vaccine in-vitro by HI test without boosting. They found that the antibody titer increased till reach its peak (6.24 log₂) at 5th week post vaccination. On the other hand, (23) evaluated a reverse genetics H5N3 avian influenza virus cell cultured vaccine by single dose and the HI titer reached its peak (6.2 log₂) at the 4th week. (21) Found that chickens which received one dose of rHA- H5 vaccine had a markedly poor response and priming by two dosages appeared to improve the antibody response. (24) evaluated inactivated H9N2 vaccine with gel-primed and mineral oil- boosted regimen in broiler breeders. They concluded that a single dose regimen of inactivated H9N2 vaccine provided not enough anti body level in the broiler breeder flock and recommended a two doses regimen than single dose. Also stated that a gel- primed and oil- boosted regimen might be an economical and effective vaccine strategy for poultry producers.

Regarding the challenge test, the protection percentages of birds against *P. multocida*

strains were 76.6, 70.0 and 80.0 %, among broiler breeder, broiler and turkey groups respectively when challenged 3weeks after single dose, while they were 90.0, 86.6 and 93.3 %, among the same groups when challenged 3 weeks after booster dose as shown in Table (3).

The results obtained by (25) who reported that the protection % in ISA 206 combined FC and ND vaccine was 100%. Also (26) noticed that chickens vaccinated with FC vaccine containing ISA 206 showed 90% protection. From these data it was clear that the antibody titer was measured with ELISA test was highly correlated with protection against challenge with virulent organisms as reported by (15).

For the protection against AI H9N2 virus, it was 83.3, 80.0 and 86.6% among the broiler breeder, broiler and turkey bird groups when challenged 3weeks after single dose as shown in Table (4). These protection percentages were raised up to 93.3, 90.0 and 96.6 % for the same bird groups when challenged 3 weeks post booster of vaccination.

An inactivated ISA 70 H9N2 vaccine was evaluated by (27) and concluded to this vaccine induced protection 100% 3weeks post vaccination where the virus isolated from 0/7 cloacal samples collected on 1, 3 and 5 days post challenge. In contrast (23) concluded that ISA 206 adjuvant vaccine provides only 50% or even less protective efficiency to SPF chickens. Meanwhile (28) recorded that the protection levels were found to increase up to 90 % when the Montanide ISA 70 and Montanide ISA 206 formulations were used.

The findings of this study indicated that the vaccine is valid and has a satisfactory result according to specifications of OIE (14).

Conclusion

Conclusively, it is very beneficial to apply the inactivated polyvalent *Pasteurella multocida* and AI H9N2 vaccine for the protection against both FC and AI outbreaks as it reduce production costs, for the administrator as it save time, effort and simplify the immunization schedule. Also for the birds, it minimizes stress of multiple vaccinations.

Table 1: *P. multocida* ELISA mean titer of different bird flocks vaccinated with the prepared FC and AI (H9N2) vaccine post single and booster dose vaccination

Type of Birds	Weeks post vaccination	Weeks post vaccination							Control
		1	2	3	4	5	6	7	
Broiler breeder	Single dose	523	841	1011	1176	1247	1208	1185	36
	Booster dose				1381	1566	1832	1792	41
Broiler	Single dose	469	719	904	1066	1191	1174	1081	39
	Booster dose				1218	1466	1578	1511	38
Turkey	Single dose	497	819	973	1118	1226	1211	1172	44
	Booster dose				1341	1494	1801	1716	29

Table 2: AI H9N2 HI mean titer of different bird flocks vaccinated with the prepared FC and AI (H9N2) vaccine post single and booster dose vaccination

Type of Birds	Weeks post vaccination	Weeks post vaccination							Control
		1	2	3	4	5	6	7	
Broiler breeder	Single dose	3.6	4.8	6.4	6.6	7.8	7.6	7.2	0.0
	Booster dose				7.2	8.2	8.6	8.2	0.0
Broiler	Single dose	3.2	4.0	5.8	5.8	7.2	6.8	6.2	0.0
	Booster dose				6.8	7.4	8.0	7.6	0.0
Turkey	Single dose	3.6	4.6	6.4	6.2	7.4	7.2	6.8	0.0
	Booster dose				7.2	7.6	8.2	7.8	0.0

Table 3: Protection percentages obtained in birds vaccinated with single and booster doses of the prepared FC and AI vaccine against virulent *P. multocida* strain

Type of Bird	Challenge 3 weeks post						Control		
	Single dose vaccination			Booster dose vaccination			Bird No	M.	P %
	Bird No	M.	P %	Bird No	M.	P %			
Broiler breeder	30	7	76.6	30	3	90.0	15	12	20.0
Broiler	30	9	70.0	30	4	86.6	15	14	07.0
Turkey	30	6	80.0	30	2	93.3	15	13	13.4

M = Mortalities P% = protection percent

Table 4: Protection percentages obtained in birds vaccinated with single and booster doses of the prepared FC and AI vaccine against AI H9N2 virus

Vaccination	Type of bird	ECE No.	HA		Protection %
			Positive	Negative	
Single dose	Broiler breeder	30	5	25	83.3
	Broiler	30	6	24	80.0
	Turkey	30	4	26	86.6
Booster dose	Broiler breeder	30	2	28	93.3
	Broiler	30	3	27	90.0
	Turkey	30	1	29	96.6

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Glisson, J.R., C.L. Hofacre and J.P. Christensen. Fowl cholera. In: Diseases of poultry, Saif YM, Barennes HJ, Glisson JR, Fadly AM, McDougald LR and Swayne DE (Editors). Blackwell Publishing, Ames, Iowa, USA, 2008; 739–58.
2. Swayne, D.E. and D.A. Halvorson. Avian influenza. In: Saif, Y.M., Barnes, H.J., Fadly, A.M., Glisson, J.R., McDougald, L.R., Swayne, D.E. (Eds.). Diseases of Poultry. 11th ed. Iowa State Press, Iowa. 2003; pp. 135–60.
3. Adler, B., D. Bulach, J. Chung, S. Doughty, M. Hunt, K. Rajakumar, M. Serrano, A. Van Zanden, Y. Zhang and C. Ruffolo. Candidate vaccine antigen and genes in *Pasteurella multocida*. J. Biotech. 1999; 73(2-3): 83–90.
4. Takai, H., Y. Hayakawa, E. Shintani, H. Komae, Y. Yoshida, H. Ide, G. Abe and T. Sawada. The first outbreak of *P. multocida* infection in turkeys in Japan. J. Japan Vet. Med. Association, 1994; 47:923–7.
5. Voyles, B.A. Orthomyxoviruses. In: The biology of viruses 2nd Ed. New York, NY: McGraw-Hill, 147. 2002.
6. Alexander, D. J. Should We Change the Definition of Avian Influenza for Eradication Purposes? Avian Diseases. 2003; 47: 976–81.
7. Bano, S., K. Naeem and S.A. Malik. Evaluation of Pathogenic Potential of Avian Influenza Virus Serotype H9N2 in Chickens. Avian Diseases. 2003; 47:817–22.
8. Alexander, D. J. A review of avian influenza in different bird species. Vet. Microbiol. 2000; 74 (1-2): 3–13.
9. Orabi A, Hussein A, Saleh A A, El-Magd M A, Munir M. Evolutionary insights into the fusion protein of Newcastle disease virus isolated from vaccinated chickens in 2016 in Egypt, Archives of Virology 2017; 162(10): 3069–79
10. Reddy, G.S.; K. Ananda and V.A. Srinivasan. Performance of oil adjuvant combined vaccine containing FMD, Rabies, *Pasteurella multocida* and *Clostridium chauvoei* antigens. Indian Veterinary Journal, 2001; 78(11): 990–3.
11. Swayne, D.E.; D.A. Senne and C.W. Beard. Influenza, in: Swayne, D.E.; Glisson, J.R.; Jackwood, M.W.; Pearson, J.E. and Reed, W.M. (Eds), Isolation and identification of avian pathogens: Kennet Square: American Association of Avian Pathologist. 1998; 150–5.
12. Villegas, P. 1998. Titration of biological suspensions. In: Swayne, D.E. (Ed), A Laboratory Manual for the Isolation and Identification of Avian Pathogens, fourth ed. American Association of Avian Pathologists, Kennett Square, PA, 1998; 248–54.
13. Sarachai, C.; J. Sasipreeyajan, and N. Chansiripornchai. Avian Influenza virus (H5N1) inactivation by Binary Ethylenimine. Thai J. Vet. Med. 2010; 40(1): 41–6.
14. OIE. Manual of Standards of Diagnostic Tests and Vaccines for Terrestrial Animals Safety Test, 2017; chapter, 2.3.4., Avian influenza page no.446.
15. Jabbari, A. R. and G.R. Moazeni Jula. Fowl cholera: Evaluation of a Trivalent *Pasteurella multocida* Vaccine Consisted of Serotypes 1, 3 and 4. Arch. Razi Ins. 2005; 59: 103–11.
16. Kwon, J.S.; H.J. Lee, D.H. Lee, Y.J. Lee, I.P. Mo, S.S. Nahm, M.J. Kim, J.B. Lee, S.Y. Park, I.S. Choi and C.S. Song. Immune response and pathogenesis in immunocompromised chickens in response to infection with the H9N2 low pathogenic avian influenza virus. Virus Research, 2008; 133: 187–94.
17. Stone, D.; M. Brugh, S.R. Hopkins, H.W. Yoder and C.W. Peard. Preparation of inactivated oil emulsion vaccines with avian viral or Mycoplasma antigens. Avian Dis., 1978; 22: 666–47.
18. Parvin, M.S., M. P. Siddique and M. T. Islam. Humoral immune response to fowl cholera vaccine in different breeds of commercial birds. Bangl. J. Vet. Med. 2011; 9 (2): 127–31.
19. Iqbal, M.T.; M.H. Haque, S. Sarker, M.A. Islam and K.A. Choudhury. Determination of immune response against alum-precipitated fowl cholera vaccine in the quail, *Coturnix japonica*. Univ. J. Zool. Rajshahi. 2010; 29: 57–60.
20. Belloc, C.; L. Dupuis, S. Deville, H. Aucouturier and A. Laval. Evaluation of safety and immune response induced by several adjuvants included in *Pasteurella multocida* vaccines in chickens. Revue Méd. Vét., 2008; 159(7): 371–5.
21. Lin, Y. J.; M. C. Deng, S. H. Wu, Y. L. Chen, H. C. Cheng, C. Y. Chang, M. S. Lee, M. S. Chien and C.C. Huang. Baculovirus-Derived Hemagglutinin Vaccine Protects Chickens from Lethal Homologous Virus H5N1 Challenge. J. Vet. Med. Sci. 2008; 70(11): 1147–52.
22. Rajabi, Z.; H.T. Nasrabadi and A.B. Syofi-Khojin. Evaluation the quality of oil-Emulsion

Avian Influenza subgroup H9N2 vaccines in vitro. *Vaccinology*, 2010; 2: 22–5.

23. Liu, C.G.; Ming Liu, Fei Liu, Da F Liu, Yun Zhang, Wei Q Pan, Hao Chen, Chun H Wan, En C Sun, Hong T Li, and Wen H Xiang. Evaluation of several adjuvants in avian influenza vaccine to chickens and ducks. *Virology*; 2011; 8: 321.

24. Lee, D.H.; J.S. Kwon, H.J. Lee, Y.N. Lee, W. Hur, Y.H. Hong, J.B. Lee, S.Y. Park, I.S. Choi and C.S. Song. Inactivated H9N2 avian influenza virus vaccine with gel-primed and mineral oil-boostered regimen could produce improved immune response in broiler breeders. *Poultry Science*, 2011; 90: 1020–2.

25. Elham, A. Youssef; Eman, A. Hassan; Mervata, A. El-Koffy and A.M. Daoud. Trial for preparation of combined oil inactivated vaccine against Newcastle and Fowl Cholera diseases by using Montanide ISA 206 oil for improving the immune response in chickens. *Vet. Med. J. Giza*, 2005; 53 (2): 341–52.

26. Hala, A.F.; I.S. Ibrahim, H.G. Fawzy and S.M. Aboul Saoud. Preparation and evaluation of avian cholera oil emulsion vaccine using Montanide ISA 206. *Egypt. Vet. Med. Assoc.*, 2002; 62(2).

27. Jun G. C.; J.L. Youn, J.K. Yong, K.L. Eun, O.M. Jeong, W.S. Haan, H.K. Jae and H.K. Jun. An inactivated vaccine to control the current H9N2 low pathogenic avian influenza in Korea. *J. Vet. Sci.*, 2008; 9 (1): 67–74.

28. Dingu, B.; B. Brett, R. Macdonald, S. Deville, L. Dupuis, J. Theron and R.R. Bragg. Study on the efficacy and safety of different antigens and oil formulations of infectious coryza vaccines containing an NAD-independent strain of *Avibacterium paragallinarum*. *Onderstepoort Journal of Veterinary Research*, 2009; 76:299–309.

29. Fatma Elzahraa G. AboElkhir, Afaf A. Khedr and Elseedy F R. Identity of *Pasteurella multocida* and Avian Influenza H9N2 Strains Used in Preparation of a Combined Inactivated Vaccine Using PCR. *Global Veterinaria* 2005; 14 (4): 535–8.