PREPARATION AND COMPARATIVE EVALUATION OF FOUR DIFFERENT OIL ADJUVANTED VACCINES AGAINST AVIAN INFLUENZA DISEASE

S. A. Chughtai [1], S. Hussain[2], W. Shahzad[1], M. S. Hussain[1], N. Mustafa[1], A. Mehmood[1] and S. Shaukat[1]

[1]Veterinary Research Institute, L&DD Punjab, Lahore
[2]Director, Veterinary Research Institute, L&DD Punjab, Lahore

ABSTRACT: Avian Influenza virus causes one of the most devastating viral disease in poultry industry and is distributed worldwide Bird flu vaccine with H9N2 strain of avian influenza virus was prepared using four different adjuvants i.e. Eolane-130, Eolane-150, Eolane-170 and Montanide oil ISA 70 MVG. Immune response of all the vaccinated and control groups after single as well as booster doses was evaluated in layer birds through Haemagglutination inhibition test. Single dose showed poor immune response while booster doses gave better response with all the vaccines. In present study, results of Haemagglutination Inhibition Test conducted on serum samples of four vaccinated and one control group indicated that the group vaccinated with Eolane-130 showed highest level of antibodies titer (GMT=576.00) on 63rd day which later declined. While the highest antibodies level recorded in groups vaccinated with Eolane-150, Eolane-170, Montanide oil adjuvanted vaccines and control groups were 400 GMT, 422 GMT, 284.4 GMT and 4 GMT respectively. All the vaccinated groups showed 100% protection when challenged with live Avian Influenza (H9 strain) virus on 63rd day. Chicken immunized with all oil adjuvanted vaccines showed no clinical signs, disease and mortality. All the vaccinated groups showed GMT titer higher than the protective GMT titer i.e. 67.29 until the end of trial.

Keywords: Avian influenza, Eolane, Haemagglutination, Embryonated eggs, H9N2, GMT.

(Received 03.09.2021 Accepted 30.11.2021)

INTRODUCTION

Infections caused by Influenza virus occur in many domesticated and wild birds. Avian Influenza virus belongs to family of Orthomyxoviridae. This virus has eight segmented, single stranded RNA (ssRNA) genome which is negative sense. Classification of Influenza virus has three different types A, B and C, on the basis of internal proteins primarily nucleoproteins (NP) and matrix protein (M). All Avian Influenza viruses are present in type A category. The virus shows two surface glycoproteins namely hemagglutinin (H) and neuraminidase (N). Classification of virus into subtypes is done depending upon the combination / grouping of 18 HA and 11 NA molecules (Tong *et al*., 2012). Avian influenza viruses are characterized into two separate pathogenic groups: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Swayne *et al*., 2008). High economic losses have been observed during AI outbreaks since it was reported in poultry in 1994 in Pakistan (Muhammad *et al*., 2006). Increased morbidity and mortality rate in the diseased flocks results in poor production performance. The AI virus (AIV) types linked with various clinical signs and symptoms in poultry flocks in Pakistan have been identified as H7N3, H9N2 and H5N (Muneer *et al.*, 2001). The phylogenetic analysis on these H9N2 AIV isolates acquired from Saudi Arabia, Pakistan and Iran indicate a similar relationship amongst them and advocates their common origin (Banks *et al*., 2000). The epidemics of H9N2 in poultry and wild birds during 1990s were caused due to introduction of feral birds (Banks *et al.*, 2000). Variable clinical signs and symptoms of AI in poultry indicates dependency of many vital factors such as host species, age, sex, concomitant infections, immune status and microbial contamination level in the environment. In addition to high morbidity and mortality (upto 100% in some cases), infected flocks show respiratory problems with decline in egg production (Easterday *et al.*, 1997). The proper vaccination practice along with biosecurity measures is an important tool to control avian influenza infection in chickens. It is observed in several experimental researches that inactivated avian influenza (AI) vaccines are fully capable in eliciting a protective antibody response, which confers protection againsr AI virus infected birds (Bublot *et al*., 2007 ; Capua *et al.*, 2008). Due to its (LPAI H9N2) widespread nature and zoonotic potential, vaccination of susceptible birds with inactivated oil adjuvanted vaccine is one of the best strategy to control the disease (Nili *et al*., 2003 ; Iqbal *et al*., 2008). Regarding effectiveness of H9N2 inactivated oil adjuvanted commercial vaccine being inoculated in Pakistan, limited scientific data is available. A 0.3 ml vaccine dose of AI (H9N2) in layer is advised to be administered as early as 7 days of age (with a booster dose) to obtain consistently high antibody titers

(Iqbal *et al*., 2008). The Montanide and Eolane adjuvants have been widely used in veterinary practice (Walid *et al.,* 2016 ; Afroz *et al.,* 2016). Both mineral oil - based adjuvants has shown in multiple studies to improve cellular immune response with insignificant side effects.

The current investigation was designed to study the comparative efficacy of four new vaccines prepared from inactivated H9N2 virus, utilizing Eolane-130, Eolane-150, Eolane-170 adjuvants and Montanide oil ISA 70 MVG. Comparative evaluations were made in terms of immunity conferred in the bird against LPAI H9N2 and safety against challenge with live virus.

MATERIALS AND METHODS

Master Seed Preparation: Avian Influenza (H9) virus isolated from poultry flocks in Pakistan was used for manufacturing of AI vaccine. Chicken embryonated eggs (9-11 days old) were used for master seed preparation 0.2 ml inoculum of (H9 strain) 10^3 to 10^4 EID_{50} was inoculated in allantoic cavity embryonated eggs (OIE, 2018) and incubated at 37° C for 72 hours with 80% relative humidity. Eggs showing dead embryos after 12 hours on candling were discarded. Harvesting was done after 72 hours of inoculation. Sterility test and Haemagglutination Test were done to confirm purity of seed. Master seed was lyophilized in aliquots and stored in frozen form at -80 to -120° C. Master seed was also confirmed from UDL UVAS Lahore.

Working Seed Preparation: Working seed was prepared from master seed and for this purpose minimum of three passages of master seed were performed. Sterility tests were done to confirm purity of seed. HA test was performed and EID_{50} was calculated. Aliquots of seed were prepared for further use which were stored at -120°C (OIE, 2018).

Preparation of Oil Adjuvanted vaccines: For this purpose initially antigen was prepared by inactivating the virus from harvested amnioallantoic fluid of inoculated chicken embryonated eggs. Inactivation was done by 0.4% Formalin (Merck Germany®) according to OIE manual 2018. Thiomersal (Bio world, USA) as a preservative was added as 0.003% in solution form. After inactivation the material was inoculated in chicken embryonated eggs (9 days old) for 48 hours to check inactivation of virus. Four oil emulsion vaccines were prepared through emulsification (10,000 rpm to 15,000 rpm) with homogenizer (IKA-Labortechnik, Germany®) using equal volumes of viral suspension ($10^{8.6}$ EID_{50}/ml) and adjuvants i.e. Eolane-130 (AI-Flu-A), Eolane-150 (AI-Flu-B), Eolane-170 (AI-Flu-C) by Total, Parco, Pakistan® and Montanide Oil ISA 70 MVG (AI-Flu-D) by Seppic, France®. Lanolin was used for emulsification (4%) in Eolane adjuvants. Span 80 was used as emulsion stabilizer and surfactant at the rate of 4% in Eolane adjuvants. The mixtures were stored overnight, re-emulsified again and stored at 4°C for 10 days (OIE, 2018 ; Shahzad *et al.,* 2020). During emulsion preparation, the temperature of the emulsified mixtures was kept between 15^0C and 18^0C. (OIE, 2018).

Stability testing: For this purpose oil adjuvanted vaccines were maintained under different storage conditions i.e. at 4°C ±0.1 °C (refrigerator) and 25°C ±0.1°C (room temperature). The following quality tests were performed after 24 hours, 14 days and 90 days (OIE, 2018).

a. **Organoleptic characteristics:** These characteristics include colour, liquefaction and phase separation of oil adjuvanted vaccines were noted after 24 hours, 14 days and 90 days at 4°C ±0.1 °C (refrigerator) and 25°C ±0.1 °C (room temperature). (Shahzad *et al.*, 2020; Kumar *et al.*, 2015)

b. **Centrifugation test:** All vaccines samples were centrifuged at 10,000 rpm to 15,000 rpm to see any phase separation after 24 hours, 14 days and 90 days at 4°C ±0.1 °C (refrigerator) and 25°C ±0.1 °C (Shahzad *et al.*, 2020; Kumar *et al.*, 2015).

c. **Drop test:** To perform this test a drop of each vaccine was poured in a glass beaker of cold water and was checked for stay at the surface. (Aucouturier *et al.*, 2001; Shahzad *et al.,* 2020)

d. **Sterility test:** A sample from each oil adjuvanted vaccine was cultured on different testing media which include Nutrient agar slant, MacConkey agar slant, Sabouraud agar slant, Thioglycolate medium and Nutrient broth. Incubation was done at 37°C for 7 days (OIE, 2018). Cultured media were observed regularly for any growth. Absence of any growth on media indicated that all vaccines samples were sterile (OIE, 2018).

e. **Safety Test:** A double dose i.e. 0.6 ml of each vaccine was administered via subcutaneous route in 10 birds (three weeks old) and kept under observation upto two weeks. Vaccinated birds along with control group were observed for development of any untoward reaction (i.e. local lesion, any clinical sign of disease) for 14 days (OIE, 2018; Shaukat *et al.,* 2016)

f. **Potency Test:** Potency test for the manufactured AIV vaccines was evaluated by its ability of eliciting sero-conversion in the experimentally inoculated chicks (Shaukat *et al.,* 2016). The immune status of each of four vaccinated groups of birds (layer) maintained at VRI (40 in each group) were estimated by Haemagglutination (HA) (OIE, 2018) and Haemagglutination Inhibition (HI) tests (Shaukat *et al.,* 2016).

Birds and experimental design: A total of 200 day-old layer chicks were randomly distributed into five groups (A, B, C, D, E) including four (A, B, C, D) experimental and one (E) control group (40 chicks / group). Identification of groups was ensured by using different color markers.

Avian Influenza Virus Vaccination: At one week of age, each experimental chick was inoculated with AIV vaccines as per following experimental design:

Group A: AI-AI-Flu-A vaccine.

Group B: AI-AI-Flu-B vaccine.

Group C: AI-AI-Flu-C vaccine.

Group D: AI-AI-Flu-D vaccine.

Group E: Controls (Unvaccinated)

Collection of blood samples: Blood samples from experimental chicks in each group were collected at day 0, 21, 35, 49, 63, 79, 93, 107 and 121 post inoculation for determining HI antibody production titer against the vaccinated AI viruses. For this purpose 2 ml blood was taken from a bird with 5cc sterilized disposable syringes. Syringes were kept in slant position at room temperature, overnight. The serum was separated in 1ml labeled eppendorf tubes and placed at -20°C for further use. These serum samples were used in HI test to check for the presence of antibodies against AIV (H9) as per procedure described by Beard and Thayer (1998). Briefly, the HI antibody titrations were conducted using H9 AIV. Four HA units of virus suspension were used for conducting HI test with serum samples collected at day 7, 14 and 21 respectively, using 1% chicken RBC suspension. The HI controls for negative and positive H9 anti-sera. All titrations were carried at room temperature i.e. 22-25 °C.

Haemagglutination Inhibition Test: The test was performed in 96 well round bottom titration plates. The Haemagglutination inhibition (HI) serological test was carried out according to the World Organization for Animal Health Manual (OIE, 2018) to check the post-vaccination humoral immune response for each vaccine type using the homologous HA antigen. A two fold serial dilution of sera were mixed with four Haemagglutination (HA) units of H9N2 AI antigen. The HI titer was determined using 1% washed red blood cells suspension, which was collected from at least three chickens, 8 week of age (Walid *et al.,* 2016). The HI titer was determined by observing the highest serum dilution preventing Haemagglutination. The geometric mean titers (GMT) of different groups were calculated according to the procedure described by Villegas and Purchase (1980).

Table 1. Vaccination schedule of Vaccinated and Control Groups.

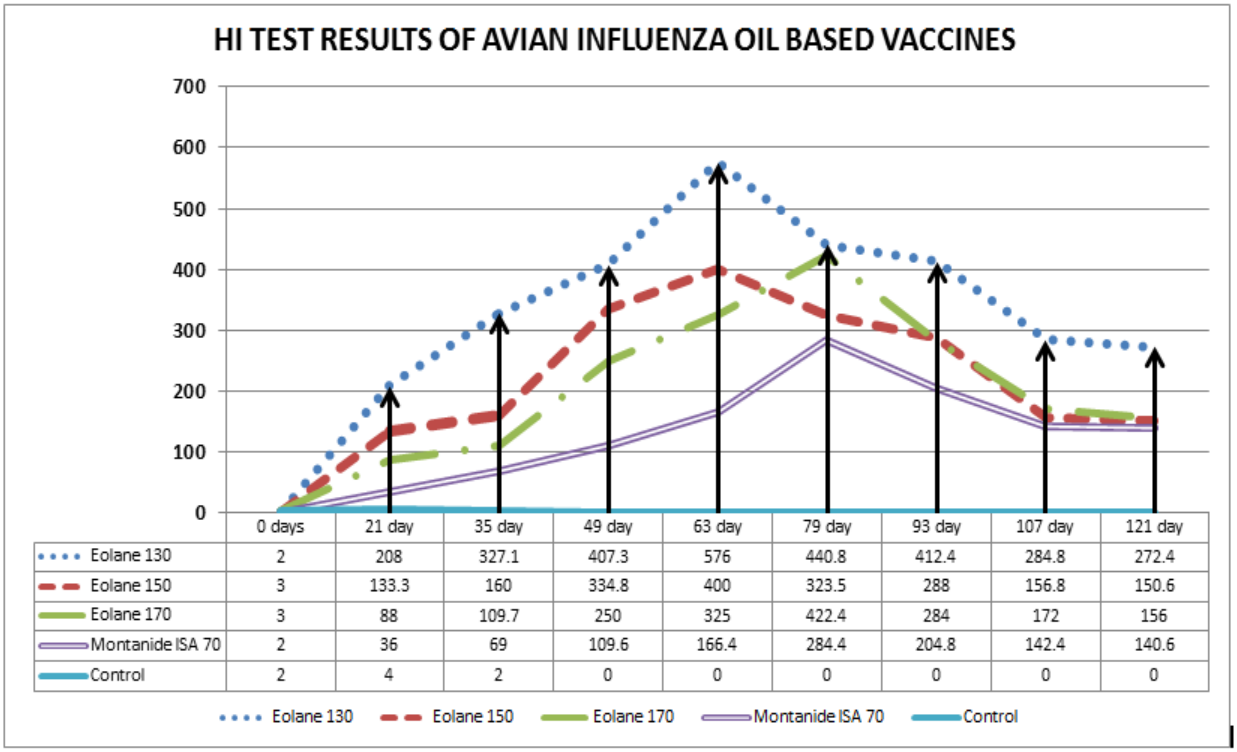
Vaccination (Age in days)	Name of vaccine	Groups Name	Route Used	Dose (ml)
Day 3	ND (Lasota-Live)	A, B, C, D, E	Drinking water	
Day 7	AI-Flu-A	A	S/C	0.3
	AI-Flu-B	B	S/C	0.3
	AI-Flu-C	C	S/C	0.3
	AI-Flu-D	D	S/C	0.3
	Normal Saline	E	S/C	0.3
Day 28 1st Booster dose	AI-Flu-A	A	S/C	0.3
	AI-Flu-B	B	S/C	0.3
	AI-Flu-C	C	S/C	0.3
	AI-Flu-D	D	S/C	0.3
	Normal Saline	E	S/C	0.3
Day 42 2nd Booster dose	AI-Flu-A	A	S/C	0.3
	AI-Flu-B	B	S/C	0.3
	AI-Flu-C	C	S/C	0.3
	AI-Flu-D	D	S/C	0.3
	Normal Saline	E	S/C	0.3

Day 3 ND lasota live was given in drinking water later at 7 ,28 and 42 day flu vaccine H9N2 was injected via subcutaneous route with a dose of 0.3 ml / bird.

Challenge Protection: A total of 5 number of birds from each experimental group were separated randomly and housed in a different experimental room for the purpose of performing post vaccination challenge protection test. The birds were challenged with homologous LPAI H9N2 virus at day 63 after second booster and kept under observation for upto 10 days. No clinical signs and symptoms were detected in all vaccinated groups but chicken from the control group showed nasal discharge,

sneezing and coughing and died day 5 post challenge with virus.

Results: All the oil based vaccines adjuvanted with Eolane-130, Eolane-150, Eolane-170 and Montanide oil ISA 70 MVG were persistently stable for upto 90 days when kept at 4°C and 25°C i.e. no change in liquefaction, colour and phase separation was observed. Centrifugation test after 90 days showed no phase separation and drop test also showed no disintegration for water in oil emulsion. Vaccines were sterile and safe in birds as per OIE (2018). Haemagglutination Inhibition Test conducted on serum samples of four vaccinated and one control group indicated that the group vaccinated with Eolane-130 showed highest level of antibodies titer (GMT=576.00) on day 63 which later on declined. While the highest antibodies level showed by groups vaccinated by Eolane-150, Eolane-170, Montanide oil adjuvanted vaccines and control groups were 400 GMT, 422GMT, 284.4 GMT & 4GMT respectively. All the vaccinated groups showed 100% protection when challenged with live Avian Influenza (H9 strain) virus on 63rd day. Chicken immunized with all oil adjuvanted vaccines showed no clinical signs, disease and mortality.



	0 days	21 day	35 day	49 day	63 day	79 day	93 day	107 day	121 day
Eolane 130	2	208	327.1	407.3	576	440.8	412.4	284.8	272.4
Eolane 150	3	133.3	160	334.8	400	323.5	288	156.8	150.6
Eolane 170	3	88	109.7	250	325	422.4	284	172	156
Montanide ISA 70	2	36	69	109.6	166.4	284.4	204.8	142.4	140.6
Control	2	4	2	0	0	0	0	0	0

Priming 7 day, 1st booster 28 day, 2nd Booster: 42 day

DISCUSSION

Outbreaks of Avian Influenza viruses give rise to heavy commercial losses in poultry industry ever since first reported in Pakistan in 1994 (Muhammad *et al.*, 2006). The infected birds showed losses by poor growth, low egg production, high morbidity and mortality, retraction of other respiratory diseases (Shaukat *et al.*, 2016).

The AI virus types associated with variable clinical signs in Pakistan have been identified as H7N3, H9N2 and H5N1 (Muneer *et al.*, 2001; Shaukat *et al.*, 2016). Vaccination along with biosecurity measures are important tools for prevention and control of Avian Influenza disease in poultry. Inactivated oil adjuvanted Avian Influenza vaccine provide better immune response in chickens compared with alum precipitated and aluminium hydroxide gel adjuvanted vaccines (Iqbal *et al.*, 2008). The oil adjuvanted vaccines form a deposit of antigen and allows for the slow release of it over long period of time. It avoids the swift degradation of antigen by enzymes and recruits the antigen presenting cells (APCs) at the site of inoculation facilitating uptake of antigen by the APCs mainly macrophages and dendritic cells (Aucouturier *et al.*, 2007). During this trial all the oil adjuvanted vaccines proved stable and no change in colour, liquefaction, phase separation was seen when stored at 4°C $\pm$0.1 °C and 25°C $\pm$0.1 °C for 24 hours, 14 day and 90 day period. No phase separation was observed during centrifugation test (Shahzad *et al.*, 2020; Kumar *et al.*, 2015).

In present study four oil adjuvants with Eolane-130, Eolane-150, Eolane-170 and Montanide oil ISA 70 MVG were used to prepare oil adjuvanted vaccines and their efficacy were compared. The Montanide ISA 70 MVG and ISA 71 MVG (SEPPIC, FRANCE) are widely used in veterinary vaccines and has demonstrated to increase the cellular immune response with nominal side effects associated with other mineral oils (Walid *et al.,* 2019).

Eolane-130, Eolane-150 and, Eolane-170 adjuvants are widely used for vaccine production worldwide and are in compliant with the Food and Drug Administration (FDA). They meet the standards of USA, EU and Japanese pharmacopoeias (Total FLUIDS, France). The vaccines prepared with Eolane adjuvants range is homogeneously dispersed showing maximum emulsion stability, decreased viscosity, easily injectable, negligible to none adverse effects and economical (Waseem *et al.,* 2007). The comparative evaluation and effectiveness in terms of antibodies production in chicks of four adjuvanted vaccines was analyzed by Haemagglutinin (HA) and Haemagglutination Inhibition (HI) tests. Serum samples from all groups were collected at day zero, 21, 35, 49, 63, 79, 93, 107, and 121. Antibody titers with GMT (Geometric Mean Titer) values of 69.29 and higher were considered as protective for Avian Influenza vaccinated birds (Trani *et al.,* 2002). Serum samples were collected from five birds at day zero in each group. Maximum GMT value of 3.00 was observed in E-150 and E-170 groups. Birds in group E showed a value of 2 GMT. On day 21, within the vaccinated groups, maximum antibody response was observed in Group A (Eolane-130 adjuvanted vaccine group) showing GMT value 208.00 and minimum was seen in Group D (Montanide 70 MVG oil adjuvanted vaccine group) i.e. GMT 36.00. Group B (Eolane-150 vaccine group) and Group C (Eolane-170 vaccine group) gave GMT 133.3 and 88.0 respectively. A GMT value of 04 was reflected by control group. The Eolane adjuvanted vaccine groups developed positive antibody titers within 14 days after giving first shot of vaccine Group D vaccinated by Montanide oil 70 MVG showed lower than protective titer. So a full recommended dose of AI (H9N2) vaccine in broiler chicks was suggested to be inoculated as early as seven day old with a booster shot to achieve higher and standardized antibody titers (Walid *et al.,* 2019).

The antibody levels on 35^{th} day elicited by Groups A, B, C, D were 327.1, 160, 109.7 and 69.0 GMT respectively. All vaccinated groups developed protective antibody titers within 28 days of vaccination. HI antibody response of Group A, B, C and D on 49^{th} day of age showed following values of GMT i.e. 407.3, 334.8, 250.0 and 109.6 respectively. These results showed booster doses of vaccination produced quite higher levels of antibody titers. Above findings agree with the earlier reports by Iqbal *et al.,* (2008) and Walid *et al*., (2016). Booster shot with oil based Avian Influenza vaccine conferred longer immune response than single shot.

The highest antibody titers were observed on day 63 in vaccinated groups A and B i.e. 576 and 400 GMT respectively and on day 79 among groups C & D highest titers were lower than the highest value recorded in group A vaccinated with Eolane-130 oil adjuvanted vaccine. Eolane-130 is therefore highly recommended for poultry vaccine production due to its inject ability speed, excellent cold properties for cold zones and effectiveness.

All groups had higher HI antibody titers than protective GMT values 69.29 (Trani *et al.,* 2002). Eolane-130 adjuvanted vaccines showed clear cut highest antibody titers throughout the trial as compared to other adjuvants.

Conclusion: The evidence obtained by the research trials concluded that the use of oil adjuvanted vaccines provides higher protective antibody titers for a long period of time and protects against challenge by live Avian Influenza virus. Eolane-130 adjuvanted vaccine showed better results keeping based on the serology, efficacy and being cheaper formulation compared to Montanide oil 70MVG adjuvant vaccine. Eolane range of vaccines are already being used world widely; being stable, cheaper, effective, friendly to use with no adverse reaction at injection site.

REFERENCES

Aucouturier, J., S. Deville, C. Perret, Vallee, and P. Boireau. Assessment of efficacy and safety of various adjuvant formulations with a total soluble extract of Trichinella spiralis. Parasite 8: S 126-S132. 2001.

Afroz, H., S. Sattar, A. Rasool, B. Zamir, I. Haq and R. Rafique. 2016. Comparison of immunogenic effect of three oil adjuvant vaccines against Haemorrhagic Septicemia in cattle and buffalo. International Archives of Bio-medical and Clinical Research, 2: 43-48.

Banks, J., E. C. Speidel, J.W. Mccauley and D. J. Alexander (2000). Phylogenetic analysis of H7 haemagglutinin subtype influenza A viruses. Arch. Virol.; 145: 1047-1058.

Beard, C. W. and S. G. Thayer (1988). Serologic procedures. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens. 4th Ed., American Association of Avian Pathologists, Philadelphia, USA. pp: 256- 258.

Bublot, M., F. X. Le Gros, D. Nieddu, N. Pritchard, T. R. Mickle, and D. E. Swayne. Efficacy of two H5N9-inactivated vaccines against challenge with a recent H5N1 highly pathogenic avian

influenza isolate from a chicken in Thailand. Avian Dis. 51: 332-337. 2007.

Capua, I., and D. J. Alexander. Avian influenza vaccines and vaccination in birds. Vaccine 26(Suppl 4): D70-D73. 2008.

Easterday, B. C., V. S. Hinshaw, D. A. Halvrson, B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. Mcdougald and Y. M. Saif (1997). Influenza. Diseases of poultry, 10th ed. Iowa State University press: Ames, IA, USA; p.583-605.

Kumar, S., V. K. Chaturvedi, B. Kumar, P. Kumar, S. R. Somarajan, A. K. Mishra and B. Sharma.2015. Effect of alum co-adjuvantation of oil adjuvant vaccine on emulsion stability and immune responses against Haemorrhagic Septicemia in mice. Iranian Journal of Microbiology, 7(2):79-87.

Muhammad, K., 2006. Workshop on production and evaluation of bird flu vaccine. WTO Quality Control Labortries, Univ. Vet. Anim. Sci., Lahore, Pakistan.

Muneer, M. A., A. M. Bahrami, Z. Munir, I. Hussain, K. Muhammad, M. Rabbani, S. Akhtar, B. Aleem, Sultan, M. A. Tariq and K. Naeem (2001). Isolation and characterization of avian influenza (H9N2) virus from an outbreak at poultry farms in Karachi. Pakistan Vet. J. 21 (2): 87-91.

Nili, H., and K. Asasi. Avian influenza (H9N2) outbreak in Iran. Avian Dis. 47: 828-831. 2003.

OIE, 2018. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Office De International Epizootics, Paris, France, pp. 821-843.

Shahzad, W., B. Zameer, S. H. Sanghi, S. Hussain and N. Mustafa. 2020. Preparation and comparative evaluation of Haemorrhagic Septicemia vaccines using Eolane-150 and Eolane-170 as oil adjuvants for cattle and buffalo. Pak. J. Agril. Engg., Vet. Sci., 36 (1):78-84.76.

Iqbal, M., M. Nisar, Anwarul-Haq, S. Noor and Z. J. Gill. Evaluation of oil based Avian Influenza vaccine (H5N1) prepared with different concentrations of adjuvant. Pakistan Vet. J., 2008, 28(4): 205-206.

Shaukat, M., M. A. Muneer, M.U. D. Ahmad, A. Maqbool and T. M. Shaukat. 2016. Efficacy of Avian Influenza virus locally manufactured and imported vaccines. J. Anim. Plant Sci. 26(3):2016.

Swayne, D. E., D. A. Halvorson. Influenza. In: Diseases of Poultry, 12th ed. Y. M. Saif, H. J. Barnes, A. M. Fadl, J. R. Glisson, L. R. McDougald, and D. E. Swayne, eds. State University Press, Ames, IA. pp. 153–184. 2008.

Tong S., LI Y., Rivailler P., Conrardy C., Castillo D.A., Chen L.M., Recuenco S., Ellison J.A., Davis C.T., York I.A., Turmelle A.S., Moran D., Rogers S., SHI M., TAO Y., Weil M.R., Tang K., Rowe L.A., Sammons S., XU X., Frace M., Lindblade K.A., Cox N.J., Anderson L.J., Rupprecht C.E. & Donis R.O. (2012). A Distinct Lineage Of Influenza A Virus From Bats. PROC. NATL ACAD. SCI. USA, 109, 4269.

Trani LD Cordioli P, Falcone E, Lombardi G, Moreno A, Sala G, Tollis M (2002). Standardization of an inactivated H7N1 avian influenza vaccine and efficacy against high-pathogenicity virus infection. Avian Dis., 47: 1042-1046.

Villegas, P. and H. G. Purchase (1980). Titration of biological Suspensions. In: A laboratory manual for the isolation and identification of Avian Pathogens. (Eds: H. G. Purchase L.H. Arp, C. H. Demermuth and J. E. Pearson). 3rd Ed., Am. Assoc. Avian Pathologists, Univ. Pennsylvania, New Bolton Centre, Kennet Square, pp. 181-191.

Walid H. Kilany, Abdel-Hamid I. Bazid, Ahmed Ali, Ayman H. El-Deeb, Mohamed A. Zain El-Abideen, Magdy El Sayed, and Magdy F. El-Kady. Comparative effectiveness of two oil adjuvant-inactivated Avian Influenza H9N2 vaccine. Avian Diseases 60:226-231, 2019.