ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

DETERMINATION OF THE SHELF LIFE OF INACTIVATED FOWL CHOLERA VACCINE DEVELOPED FROM LOCAL ISOLATES OF *PASTEURELLA MULTOCIDA*

MSc THESIS

BY

DAWIT DHUFERA

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfilment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

By

Dawit Dufera

August, 2022

Bishoftu, Ethiopia

Approval sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

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As members of the examining board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by: Dawit Dufera entitled "Determination of the shelf life of inactivated fowl cholera vaccine developed from local isolates of *Pasteurella multocida*" and recommend it to be accepted as fulfilling the thesis requirements for the degree of Masters of Veterinary Science in Veterinary Microbiology.

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First, I declare that this MSc thesis is my work which all sources of material used for this thesis are acknowledged. This thesis has been submitted in partial fulfillment of the necessities for an advanced (MSc) degree at Addis Ababa University, College of veterinary medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under the principles of the Library. I declare that this thesis isn't submitted to the other institution anywhere for the award of any degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AGID	Agar Gel Immunodiffusion Assay
ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
CMI	Cell-mediated Immunity
DNA	Deoxyribonucleic acid
FC	Fowl Cholera
I-ELISA	Indirect Enzyme Linked Immunosorbent Assay
IHA	Indirect Hemagglutination Assay
LPS	Lipopolysaccharide
NVI	National Veterinary Institute
OD	Optimal Density
OIE	Office International des Epizooties
P. Multocida	*Pasteurella multocida*
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
S/P ratio	Sample to Positive ratio
SEM	Standard Error of Means
TSA	Tryptose Soy Agar
TSB	Tryptose Soy Broth

SUMMARY

Fowl cholera (FC) is caused by *Pasteurella multocida (P. multocida)* and is a highly contagious disease that causes very high economic losses to the poultry industry around the world through high mortality, weight loss, low production of hatching eggs, reduction of fertility and carcass condemnation. Vaccination is one of the most widely used preventative strategies in the world to minimize illness prevalence and incidence of diseases including FC. Although NVI started to produce the killed FC vaccine in 2019, the shelf-life of the vaccine at 2-8^{0}C storage conditions was not determined; this was the objective of the current study. The shelf life was determined based on an indirect approach i.e. through evaluation of immune response to the vaccine stored at different time points as a direct approach to determining antigen content was not practicable. Hence, a total of 175 layer chickens (8 weeks old Bovans Brown) hatched and grown at Research and Development Laboratory, National Veterinary Institute (NVI) were used to determine the shelf life of the formalin-inactivated alum adjuvant FC vaccines. The vaccine's shelf life was determined using primary and secondary (booster) dose immunization of chicken with formalin-inactivated alum adjuvanted FC vaccine stored at 2- 8^{0}C for two weeks, three, six, nine, twelve, eighteen and twenty-four months. Blood was collected from each chicken before primary immunization (at day zero), and on days 21 and 35 after primary vaccination to determine serum antibody levels by Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA). All chickens used for this study indicated a low cut-off OD value of 0.2 and that they were free from serum antibody response against Avian P. multocida before immunization (at day 0). The level of chicken serum antibody (IgG) titre was significantly increased after 3 weeks of the first immunization. After two weeks of the second vaccination, the titre substantially increased in all chickens. Antibody titre was increased within the group from primary vaccination to secondary (booster) vaccination. However, antibody titre was decreased among the groups with advancing storage time of the vaccine. As a result, formalin-inactivated alum adjuvant FC vaccine-induced antibody response while being kept at the standard recommended condition of storage for up to twenty-four months.

Key words: Fowl cholera, *Pasteurella multocida,* Vaccine, Adjuvant, Formalin, shelf life.

1. INTRODUCTION

P. multocida causes FC, which is a highly contagious disease that causes very high economic losses to the poultry industry around the world through high mortality, weight loss, low production of hatching eggs and reduction of fertility and carcass condemnation (Angrick *et al*., 2001; Glisson *et al*., 2008). This bacterium is known as "multocida," which can be translated as a bacterium that "kills" (cida) "many" (multo). Pasteur was able to cultivate this bacterium in 1879, marking the first time disease-causing germs were cultivated outside of the animal host on culture media. Pasteur accidentlly discovered that inoculating hens with old broth cultures that had been attenuated protected them from *P. multocida* challenge. These were the first studies with bacterial vaccinations that were documented (Huberman, 2016).

P. multocida is a gram-negative, non-motile, cocco-bacillary shaped organism that lives in the respiratory tracts of many wild and domestic birds and causes sickness (Harper *et al*., 2006). *P. multocida* can be divided into five capsular types (A, B, D, E and F) based on capsular antigens and 16 (1 to 16) somatic serotypes based on lipopolysaccharide (LPS) antigens using capsule serogroup antigens (Christensen *et al*., 2005). Avian Pasteurellosis isolates from avian species are predominantly serotyped A, commonly known as FC which causes high mortality and morbidity rates in infected farms (Rhoades and Rimler, 1989). The mortality rate of poultry due to disease is believed to be between 20% and 50%, but during epidemics in Ethiopia, it can reach as high as 80% and one of the key issues restricting Ethiopian chicken production (Aberra and Tegegne, 2007). The disease is widespread and endemic in most areas of Ethiopia, resulting in severe economic losses due to decreased productivity and mortality (Molalegne *et al*., 2009).

P. multocida affects all bird species, while turkeys may be the most seriously impacted. FC is most usually detected in mature chickens over the age of 16 weeks, while it is rarely observed in young chicks under the age of 8 weeks (Petersen *et al*., 2001; Glisson *et al*., 2008). Because of age differences, the disease is seen more commonly in layers than in broilers (Sander and Glisson, 1989). Chickens, turkeys, pheasants, pigeons, waterfowl, sparrows, and other wild, free-flying birds are all susceptible to this widespread bacterial disease. It causes an increase in mortality in the short term. There is a chronic variant of

the disease that can develop after an acute outbreak. It causes lameness, swollen wattles (in chickens), pneumonia (in turkeys), and torticollis in the long term, but it can sometimes go unnoticed (Tabler *et al.*, 2019).

FC can be avoided by removing any infection reservoirs and then preventing the organism from returning to the property. FC can be prevented by implementing standard good management practices, an adequate sanitation system and a good biosecurity program (Blackall, 2003). Vaccination is one of the most widely used preventative strategies in the world to minimize illness prevalence and incidence (Kardos and Kiss, 2005). Vaccines, both live and inactivated, have been tried to control the disease (Glisson *et al*., 2008). Inactivated vaccinations are the most common, as the organisms have no potential of reverting to virulence and causing disease (Hopkins and Olson, 1997). Although live attenuated vaccines provide good protection with a long duration of immunity and cross-protection against different serotypes of *P. multocida*, they are less widely used in many countries due to side effects, reactions such as organism localization in joints and the potential for a lung infection, and the main one is the lack of regular maintainable attenuation or instability, with the risk of the organism regaining its virulence (OIE, 2004).

To provide strong protection, it is essential and desirable to produce vaccines from locally circulating strains. Formalin inactivated alum adjuvant FC vaccine that provided effective antibody tites against FC was developed from a local strain of *P. multocida* in Ethiopia (Wubet *et al.*, 2019). The protective parameters are evaluated by vaccination and challenge experiment, which is used to assess the vaccine's efficacy (OIE, 2017). Immune responses differ depending on the breed and the growing area (Rana *et al*., 2010). The immunological response varies greatly in younger chickens (1-5 weeks of age), and birds vaccinated at 1 or 2 weeks of age appear to be consistent with a relatively low humoral antibody response (Dick and Avakian, 1991). The immune system protects against infectious diseases, and the humoral immune response, which is driven by serum antibodies generated by B cells, is one of the most important immunological defense mechanisms (Weigend *et al*., 1997). Serological testing is a valuable tool for determining a bird's immunological status, and the I-ELISA is used to detect antibodies against *P. multocida* using a variety of antigens such as outer membrane protein (Dogra *et al*., 2015).

1.1. Statement of the problem

Recently, a formalin-inactivated vaccine against FC has been developed and is being produced at NVI from local *P. multocida type A* isolates. The performance of the vaccine was evaluated using three types of adjuvants (sterile Montanide oil, Aluminium hydroxide (gel), and Aluminium potassium sulphate (Alum) where the alum adjuvanted vaccine showed superior protection (Wubet *et al.*, 2019). However, how long the vaccine remains effective if stored under the recommended temperature of $+4^0$Cconditions is not yet determined. This study is therefore carried out to fill this information gap and thus determine the optimum shelf life of the vaccine under which it remains effective.

1.2. Objectives

The objective of this study was to determine the shelf life of formalin-inactivated alum adjuvanted FC vaccine in chickens through evaluation of immune response**.**

2. LITERATURE REVIEW

2.1. Description of fowl cholera

Avian pasteurellosis and Avian haemorrhagic septicaemia are two common synonyms for FC (Wobeser, 1997). One of the most serious and significant sources of economic losses in the chicken industry is respiratory illnesses. *P. multocida* causes a septicemic respiratory complex, which is a highly prevalent and widely distributed disease in chickens and other bird species (Rhoades *et al*., 1989; Xiao *et al*., 2015). Infections with *P. multocida* cause significant losses in layer and breeder flocks in the poultry industry (Harper *et al*., 2016).

2.2. Aetiology

P.multiocida, *Pasteurella septica* and *Pasteurella gallicida* are the three subspecies of *P.multiocida*, which is considered a single species. Subspecies *P. multocida* is the most prevalent cause of FC disease, but *Pasteurella septic* and *Pasteurella gallicida* can also cause cholera-like disease (Jens, 2016). Capsular serogrouping and somatic serotyping are used to characterize *P.multiocida's* antigenic properties. FC-causing strains belong to a variety of immunotypes (or serotypes). A specific multiplex capsular polymerase chain reaction technique that enables quick and specific capsular typing has been developed (Townsend et al., 2001). Serotype A is the most common serogroup. In chickens, type A: 1 strain of FC cause 80% mortality, but type D strains of FC cause 20% mortality (Modak *et al.*, 2012).

2.3. Nature of *Pasteurella multocida*

P. multocida belongs to the *Pasteurellaceae* family and is the type species of the genus *Pasteurella*, which was named after Louis Pasteur, who first recognized *P. multocida's* importance in the etiology of FC in the 1880s and conducted immunization research with the agent. It is a non-motile, coccobacillus, capsulated, non-spore producing bacterium that can be found single, in pairs, or as chains or filaments (Ashraf *et al*., 2011; Ievy *et al*., 2013; Akhtar, 2013). *P. multocida* is an aerobic and facultative anaerobic bacterium that thrives at temperatures between 35 and 37 degrees Celsius. Primary isolation is commonly done on media like blood agar, trypticase soy agar, or dextrose starch agar, and isolation can be increased by adding 5% horse serum to these mediums. After 18–24 hours of incubation, colonies range in size from 1 to 3 mm in diameter. Grey, butyraceous, transparent, discrete, round and convex colonies are the most common characteristics (OIE, 2015).

P. multocida is a delicate organism that is readily inactivated by ordinary disinfectants, sunlight, drying, or heat, as well as ultraviolet (UV) and gamma radiation, and investigations suggest that *P. multocida* can only survive in the environment for a maximum of thirty days (Rimler and Glisson, 1997). As a result, contaminated surroundings are unlikely to act as reservoirs for longer than thirty days (Christensen and Bisgaard, 2000). The organism is surviving for at least one month in droppings, three months in decomposing bodies and two to three months in soil. *Pasteurella* enters oral and upper respiratory tract tissues and is not passed through the egg. The disease is rarely found in hens under the age of four months, although it is prevalent in turkeys of that age (Tabler *et al.*, 2019).

2.4. Virulence factors of *Pasteurella multocida*

Virulence characteristics are important in deciding whether a strain is harmful in some hosts but not in others. Capsular serogroup A strains, as well as somatic serotypes 1, 3, and 4, have been identified as the causal agent of FC (Glisson *et al*., 2013). The presence of *P.multocida's* capsule, a polysaccharide structure that is one of the most essential virulence factors for this species, aids its capacity to penetrate and reproduce within the host (Willkie *et al*., 2012). Desiccation resistance, antiphagocytic activity, and interaction with the

complement system are all functions given to the capsule (Boyce *et al.*, 2000). Because the hyaluronic acid structure is identical to that of the tissue structure of the bird, it is thought that the type A capsule, made of hyaluronic acid, may act as a mask against the immune system (Huberman, 2016).

P. multocida pathogenesis is a complicated interaction between host-specific variables and unique bacterial virulence factors; as a result, understanding disease pathogenesis is difficult and dependent on the bacterial strain, animal model, and their interactions (Harper et al., 2006). The primary virulence factors for *P. multocida* are capsule, LPS, fimbriae, dermonecrotic toxin, hemoglobin binding protein and outer membrane proteins (Ewers *et al*., 2006). These virulence factors aid in host invasion, colonization, and tissue harm (Harper *et al*., 2006) as a result, information and knowledge about virulence factors are required to prevent and control *P. multocida* infection.

2.5. Epidemiology

2.5.1. Geographical distribution

P. multocida is found worldwide distribution in more than 180 different bird species and causes respiratory and septicemic illnesses (Samuel *et al*., 2007). Both domestic and wild birds are affected by infection, which causes a severe, systemic sickness that is always fatal and highly contagious (Carver *et al*., 2013).

2.5.2. Host range

FC has been reported in a wide range of avian hosts, implying that all species of birds are susceptible. Turkeys are the most affected of all the poultry species. An infected flock's majority, if not all, of its members may die within a few days. The disease is most common in young mature turkeys, but it can affect turkeys of all ages (Glisson, 2008). Because avian isolates are largely non-pathogenic in mammals exposed via oral or subcutaneous routes, FC is not regarded to have zoonotic potential (OIE, 2018).

2.5.3. Transmission and risk factors

The presence of feather shafts in the ventricular lumen of most bird family carcasses diagnosed with FC shows that scavenging or cannibalism of FC-dead birds is a primary mechanism of transmission (Wille *et al*., 2016). The occurrence of FC can be linked to monthly mean temperature, relative humidity, and rainfall with a 2-3 month lag. Climate variability plays an essential role in FC transmission and the model of strong predictive capacity for the occurrence of FC (Qin *et al*., 2017).

The organism can enter the body through the respiratory system, which is the most common site of infection. The excretions of visibly sick or seemingly healthy carriers, as well as surviving birds from diseased flocks, are the most effective sources of infection. The organism's rapid spread is aided by excretions from the mouth, nose, and conjunctiva, which contaminate soil, water, feed, and other surfaces. Farm workers, contaminated shoes, and equipment all play role in mechanical transmission. The infection does not spread horizontally through the egg (Glisson, 2008).

Table 1**:** The potential transmission of FC to wild birds (Wobeser, 1997)

Route of transmission and field situation	Comments
Bird-to-bird contact	The transmission of FC to birds involves the shedding of *P. multocida*. This can be done by close contact with infected individuals feeding on aquatic plants.
Ingestion	The most common route of transmission for this disease is through the consumption of infected carcasses by scavenging and predatory animals.
Aerosol	This can also be spread through the activities of people and animals that come into contact with contaminated water.
Insects	Biting insects that feed on birds after consuming contaminated carcasses or being exposed to contaminated environments (ticks, mites, flies). Bird-feeding insects (maggots, flies) after ingestion of P. multocida by the insect during feeding.
Animal bites	Infection in wild birds is not regarded to be a common occurrence. Small mammal bites, such as those from raccoons, can cause *P. multocida* infections to spread throughout the body, potentially causing disease outbreaks. Although it has been observed in certain domestic turkey flocks, no evidence of it in wild birds has been found.
Fomites (inanimate objects)	*P. multocida* can be introduced through mechanical transport methods such as contaminated cages, equipment, and clothing used in field operations. *P. multocida's* environmental persistence is sufficient for this to be a factor to consider when manpower and equipment are employed to combat an FC epidemic and then redirected for other purposes.

2.6. Diagnostic techniques of fowl cholera

Based on history, observation of typical clinical indications and lesion/post-mortem lesions tentative diagnosis can be made. Where the disease is endemic, mild or chronic forms of the disease might develop, with localized infection affecting the respiratory and skeletal systems (OIE, 2015). To distinguish from other bacterial diseases, such as salmonellosis, colibacillosis, and listeriosis in chickens, and pseudotuberculosis, erysipelas, and chlamydiosis in turkeys, a definite diagnosis can only be made by bacterial culture and the isolation and identification of the organism as *P. multocida* from cases of FC (OIE, 2018).

2.6.1. Clinical signs

Depending on how far the disease has progressed, the clinical symptoms of FC can be rather diverse. There are acute and chronic types of the condition. Clinical signs such as fever, ruffled feathers, oral mucus production, diarrhoea, and increased breathing rate show only a few hours before death in the acute form. The chronic stage of the disease may come after an acute stage or may be the flock's only form of the sickness. Localized infection at the wattles, sinuses, leg or wing joints, enlarged eyes, twisted neck, rales, and pin-headed necrotic foci in the liver with a septicemic picture are all symptoms of this kind (Glisson *et al.*, 2008; Akhtar *et al.*, 2016). Outbreaks associated with more chronic symptoms are more likely to last several weeks or even months. Because of the accumulated deaths and sick birds in the laying phase, egg production will fall (Dorsey and Harshfield, 1953).

Signs and symptoms of chronic FC are usually associated with localized infections of the sternal bursa, wattles, joints, tendon sheaths, and footpads, which are often enlarged due to accumulated fibrinosuppurative exudate. Lameness, as well as exudative conjunctivitis and pharyngitis, are possible symptoms. When the meninges, middle ear, or cranial bones get infected, torticollis might develop (Christensen and Bisgaard, 2000). As the disease advances, patients with extensive pulmonary involvement will have loud respiratory rales and coughing. There may be high to very high morbidity and mortality depending on the strain of *P. multocida* involved. Some injured birds may slowly recover after a time of depression with less virulent strains. Death normally happens rapidly after a brief period of

prostration, accompanied by convulsive wing flapping and paddling, in more virulent strains. Birds that survive the acute sickness may fully recover or acquire exudative arthritis in their legs or wings (Willkie, *et al*., 2012).

2.6.2. Post-mortem lesions

Clinical symptoms and lesions associated with localized infections can arise in chronic infections. The pulmonary system and tissues connected with the musculoskeletal system are frequently the locations of chronic infection, which can range from none to a few small haemorrhages. Some of the lesions caused by FC sickness include enteritis, yolk peritonitis, focal hepatitis, purulent pneumonia (particularly in turkeys), cellulitis of the face and wattles, purulent arthritis, and lungs with a consolidated pink 'cooked' appearance in turkeys (OIE, 2008). Acute fibrinous and necrotizing lesions affecting the spleen, air sacs, and pericardium, as well as nonspecific hepatomegaly and splenomegaly, were the most common gross necropsy findings in birds with diagnosed avian cholera. Pinpoint haemorrhages in the mucous and serous membranes, as well as abdominal fat; inflammation of the upper third of the small intestine; a light, firm "parboiled" appearance to the liver and a creamy or solid collection of material in the joints are examples of typical lesions. The disease may last a long time, especially in chickens (Wille *et al*., 2016).

Table 2: shows the several types of tests that can be used to diagnose FC and what they are used for (OIE, 2018).

Method	Purpose					
	Infection-free status	Control the movements.	To eradicate	Clinical case confirmation	Prevalence of infection surveillance	post-vaccination
Identification of the agent						
Culture	–	–	–	+++	–	–
Immune response detection						
Serological ELISA	–	–	–	–	–	++

Key: +++ = recommended for this use; ++ recommended but with limitations; + = suitable in extremely limited conditions; – = not recommended for this purpose. ELISA = enzyme-linked immunosorbent assay.

2.6.3. Isolation and identification of Pasteurella multocida

It's usually simple to isolate the organism from visceral organs like the liver, bone marrow, spleen, or heart blood of birds that die from the acute form of the disease, as well as from exudative lesions in birds with the chronic form. It can be difficult to separate chronically ill birds with no signs of sickness. A sterile cotton swab, wire, or plastic loop is inserted through the heat-sterilized surface of the tissue to be cultured to acquire the specimen. The material is directly inoculated onto agar medium or into tryptose or another broth medium and then incubated at 37^0C for 18–24 hours (OIE, 2018).

P. multocida has been isolated and identified using the ability to culture or purify the bacterium in the laboratory. After that, the pure organism is categorized based on phenotypic characteristics like shape and carbohydrate fermentation patterns. However, cultural conditions can affect the expression of these characteristics, reducing the stability and reliability of phenotypic strain identification approaches (Matsumoto and Strain, 1993; Jacques *et al*., 1994).

Characteristics of colony

Primary isolation is commonly done on media like blood agar, trypticase–soy agar, or dextrose starch agar, and isolation can be increased by adding 5% heat-inactivated serum to these mediums. The supplemental serum is usually not required in maintenance media. After 18–24 hours of incubation, colonies range in size from 1 to 3 mm in diameter. Discrete, round, convex, transparent, and butyraceous are the most common characteristics. Non-capsulated organisms tend to produce larger colonies than capsuled species (OIE, 2015). The cellular morphology of suspected colonies was next examined by grams and methylene blue staining. The cells are cocco-bacillary or short rod-shaped, with a diameter of 0.2–0.4 x 0.6–2.5 µm, stain gram-negative, and can be found individually or in pairs. Bipolar staining with wright or giemsa stains or methylene blue is seen in freshly isolated organisms or those discovered in tissue smears, and they are usually encapsulated (OIE, 2018).

Biochemical characteristics

The findings of biochemical tests are used to identify the target organism. The reactions of carbohydrate fermentation are critical. Glucose, mannose, galactose, fructose, and sucrose are among the fermented carbohydrates. Rhamnose, cellobiose, raffinose, insulin, erythritol, adonitol, m-inositol, and salicin are examples of non-fermentable sugars. In most cases, mannitol is fermented and arabinose, maltose, lactose, and dextrin are not fermented. *P. multocida* does not produce haemolysis, is not motile, and grows on MacConkey agar only infrequently. Nitrate is reduced; indole and hydrogen sulfide is produced, and methyl red and Voges–Proskauer tests are negative (OIE, 2018). The tests and results listed in table 3 may usually be used to distinguish *P. multocida* from other avian *Pasteurella spp*. and *Riemerella (Pasteurella) anatipestifer*.

Table 3: Test used to differentiate *P. multocida* from other *Avian Pasteurella species* and *Riemerella anatipestifer* (OIE, 2018).

Test	*Pasteurella*		*Riemerella*
	multocida	*gallinarum*	*anatipestifer*
Haemolysis on blood agar	−	−	v
Growth on MacConkey agar	−	−	−
Indole production	+	−	−
Gelatin liquefaction	−	−	+u
Catalase production	+	+	+
Urease production	−	−	v
Glucose fermentation	+	+	−
Lactose fermentation	−u	−	−
Sucrose fermentation	+	+	−
Maltose fermentation	−u	+	−
Ornithine decarboxylase	+	−	−

Test reaction results: − = no reaction; + = reaction; v = variable reactions; −u = usually no reaction; +u usually a reaction.

2.6.4. Detection of Pasteurella multocida

Capsular serogrouping and somatic serotyping are used to characterize the antigenic properties of *P. multocida*. The indirect haemagglutination (IHA) test is used to determine capsular serogroups (Carter, 1955; 1972). A, B, D, E, and F are the capsular serogroups. Except serogroup E, all have been isolated from avian hosts. A non-serological disk diffusion test has been developed that uses specific muco-polysaccharides to distinguish serogroups A, D, and F. (Rimler, 1994). A specialized multiplex capsular PCR technique

that enables quick and specific capsular typing has been developed (Townsend *et al*., 2001).

Agar Gel Immunodiffusion Assay (AGID) test is commonly used to determine somatic serotypes (Heddleston, 1962; Heddleston *et al*., 1972). All 16 serotypes have been isolated from avian hosts; serotypes 1 to 16 have been reported (Glisson *et al*. 2013). Differentiation of the 16 somatic serotypes is possible using a specific multiplex PCR technique. It has proven to be more accurate and time-saving than traditional typing (Harper *et al*., 2015).

Serological methods

Serological tests, such as ELISA and IHA have been used to identify antibodies against *P. multocida* in poultry sera (Marshall *et al*., 1981). In addition, an IHA approach for identifying *P. multocida* capsular antigens can be established (Solano *et al*., 1983). The immunogenicity is investigated by using the IHA and ELISA tests to determine the serum antibody titer (OIE, 2015). The IHA test has previously been investigated for its potential utility as a practical approach to determining the immunological response of chickens following vaccination programs (Islam *et al*., 2017 and Sultana *et al*., 2013).

PCR techniques

Phenotypic differentiation tools have been mostly replaced by genotypic methods in recent years (Taylor *et al*., 2010). For identifying and distinguishing the strains, PCR-based typing techniques were found to be quick and accurate. PCR targeting capsular gene cap specific for *P. multocida* can be used to confirm the isolated organism as *P. multocida* (OIE, 2008).

2.7. Prevention and control

Although Pasteur in 1880 demonstrated immunity in fowls inoculated with attenuated cultures of *P. multocida*, workers since that time have had irregular results with various vaccines and bacterins. Generally, no protection was provided to the vaccinated fowls, the resulting immunity was low level and short duration (Pasteur, 1880). Immunization has

never been accepted as a dependable control measure for FC. To avoid infection, proper hygiene, rodent control, and a strict biosecurity plan are required. There are vaccines available to help manage an outbreak within a flock. Vaccination is only indicated if poultry cholera is already prevalent and causing problems on the farm (Jonas *et al*., 2001)

2.7.1. Overview of vaccine

The name "vaccine" comes from the Latin Variolae vaccinae (cowpox), which Edward Jenner demonstrated could protect people from smallpox in 1798. Today, the term "vaccine" refers to all biological preparations made from living organisms that boost immunity and either prevent (prophylactic vaccinations) or treat disease (in some circumstances) (therapeutic vaccines). Vaccines are given in liquid form and can be given via injection, oral administration, or intranasal administration. Vaccines can include the complete disease-causing microbe or just a portion of it (Jenner *et al.*, 2012)

2.7.2. The development of veterinary vaccines

Vaccinations are an efficient way to protect animals against a variety of diseases. Several efficient vaccinations have been developed in the field of vaccinology, which has greatly decreased the impact of various key diseases in companion animals and livestock (McVey and Shi, 2010; Unnikrishnan *et al*., 2012). Because they elicit both cellular and humoral immune responses, live attenuated vaccines can be quite powerful (Da Costa *et al*., 2015; Rizzi *et al*., 2012). However, a key worry related to vaccines of this type is the possibility of the microbe reverting to a virulent phenotype (Shimoji *et al*., 2002; Unnikrishnan *et al*., 2012). Vaccinations that have been killed or inactivated are generally safer, but they may be less effective than attenuated vaccines (Dunham, 2002; Redding and Weiner, 2009).

Historically, veterinary vaccines were developed using empirical trial-and-error methods, to reproduce the immunity conferred by natural infection through vaccination (Doolan *et al*., 2014). The traditional "isolate, kill and inject" method can protect against a variety of bacterial and viral diseases. Live attenuated, killed/inactivated microorganisms and toxoids make up the vast majority of licensed veterinary vaccines today. In reality, the widespread use of these vaccinations has made a significant contribution to animal and public health improvement (Delany *et al*., 2014).

Currently, inactivated vaccines are made up of bacterins from one or more bacterial species or serotypes, or killed viral strains, which are usually mixed in an oil or aluminium hydroxide adjuvant (Meeusen *et al*., 2007). Inactivated vaccinations are more stable in the field and less costly to manufacture than live vaccines (Van Gelder and Makoschey, 2012). Inactivated vaccines have better safety profiles, but because of the destruction of pathogen reproduction, they cannot provide effective long-term protection (Cho, Howard and Lee, 2002).

Table 4: Characteristics of vaccines available for veterinary use (Jorge and Dellagostin, 2017)

Type of vaccines	Characteristics
Live-attenuated	Live strains are not highly protective; Reversion to virulence to a more virulent phenotype can occur; Need for refrigerated storage; Induce good both cellular (CMI) and humoral (antibody response IgG) immunity; Low stability; short shelf life, poor safe; low dose, good efficacy and long-lasting duration of immunity; no need adjuvants.
Inactivated(killed)	Inactivated vaccines offer good safety profiles; Can't provide effective long-term protection due to the destruction of the pathogen replication; Many inactivated vaccines are unable to cope with the prevailing strains in the field; Frequently, new vaccines have to be generated from field strains with new outbreaks; high stability; long shelf life; not reversion of virulence; high dose; poor efficacy; need adjuvants; IgA and IgG antibody response; poor CMI.
Toxoids	The amount of toxin produced in vitro is unpredictable; High levels of biosafety are required.
Recombinant subunit	Well-defined composition; No risk for pathogenicity; Can be produced in a variety of protein expression systems; Possibility for cost-efficient production and purification; Primarily humoral immune response; Need of adjuvant.
RNA/DNA-based	Humoral and cellular immune responses (antigen presentation by both MHC class I and II molecules); Challenges in adequate cellular uptake and expression; Long-term persistence of immunogen; The risk of host genome integration is not completely ruled out; Unstable and relatively expensive production (for RNA vaccine).
Vectored-based	Induce both cellular and humoral immune responses; In vivo amplification systems are available; Some vaccines are commercially available with a well-known safety record; Viral vectors allow for efficient infection of target cells.

2.7.3. Mechanisms of action of vaccines

Vaccines work by generating effector mechanisms (cells or molecules) that can quickly stop infections from multiplying or inactivating their toxic components. Vaccine-induced immune effectors are antibodies produced by B cells that can attach to a toxin or a disease specifically (Cooper and Nemerow, 1984). Other potential effectors include cytotoxic CD8+ T lymphocytes, which may limit the spread of infectious agents by recognizing and killing infected cells or secreting specific antiviral cytokines, and CD4+ T-helper (Th) lymphocytes, which may recognize and kill infected cells or secrete specific antiviral cytokines. These Th cells may help to defend the body by producing cytokines and assisting in the formation and maintenance of B and CD8+ T-cell responses. T-helper 1 (Th1) and T-helper 2 (Th2) subsets of effector CD4+ Th cells were initially differentiated based on their primary cytokine production (interferon- or interleukin [IL]-4) respectively (Geginat *et al*., 2014)

A key subpopulation of vaccine-induced CD4+ lymphocytes has recently been discovered. The cells are follicular T-helper (Tfh) cells, which are equipped and positioned in the lymph nodes to enable potent B-cell activation and differentiation into antibody-secreting cells (Crotty *et al*., 2015) and have been identified as influencing antibody responses and promoting adjuvanticity (Gavillet *et al*., 2015). T-helper 17 (Th17) cells are another significant fraction that mostly defends against extracellular bacteria that colonize the skin and mucosa, attracting neutrophils and causing local inflammation (Kumar *et al*., 2013). These effectors are regulated by regulatory T cells (Tregs), which are important in immunological tolerance maintenance (Sakaguchi *et al*., 2010). Most antigens and vaccinations elicit B- and T-cell responses; therefore vaccine promotes both humoral and cellular immune responses. Furthermore, most antibody responses require CD4+ T cells, whereas antibodies have a major impact on T-cell responses to intracellular infections (Igietseme *et al*., 2004).

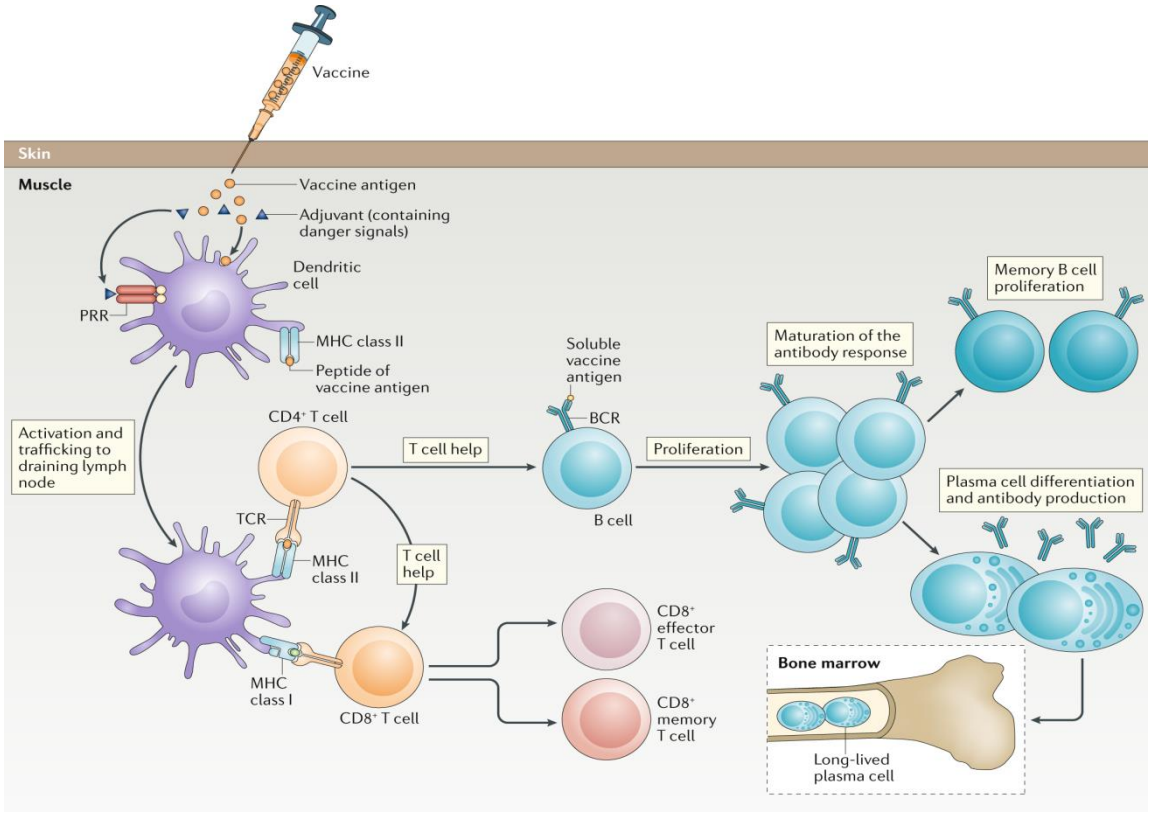

Figure 1: The generation of an immune response to a vaccine (Pollard and Bijker, 2021)

Figure 1 show the immunological response elicited after immunization with a typical protein antigen. MHC molecules on the dendritic cell present peptides of the vaccination protein antigen, which activate T cells via their T cell receptor (TCR). T lymphocytes stimulate B cell growth in the lymph node via signaling (through soluble antigen) through the B cell receptor (BCR). The maturation of the antibody response to increasing antibody affinity and inducing diverse antibody isotypes occurs as a result of T cell-dependent B cell growth. Over the next two weeks, serum antibody levels rise rapidly due to the development of short-lived plasma cells that actively generate antibodies specific to the vaccine protein. Memory B cells, which mediate immunological memory, are also created. Long-lived plasma cells, which can manufacture antibodies for decades, move to bone marrow niches to live (Pollard and Bijker, 2021).

2.7.4. Factors affecting vaccine effectiveness

A safe vaccine isn't just one that's been developed, tested, and proven safe in clinical studies. As important as those factors are, there are additional ways to make vaccinations safer. These include safe transportation and storage to the place of administration, safe administration and immediate post-marketing surveillance to detect any unexpected reactions. The potency, shelf life, efficacy, and duration of immunity of veterinary vaccines might vary depending on the producer (James, 2007).

Another vaccination component that impacts vaccine efficacy is adjuvant. Adjuvants are immunogenic chemicals that, although not providing immunity by themselves, improve the immunogenicity of the vaccines with which they are combined. The innate immune system is the body's initial line of defense against infection, and it is the arm of the immune response that is triggered by the vaccine adjuvant. As a result, adjuvant activation of this innate system is essential for successful immunization (Pulendran, 2011). To elicit a sufficient immune response, all non-living vaccinations require an adjuvant. Aluminium salts and derivatives of the glycoside saponin are among the adjuvants used in animal vaccines. Adjuvants boost immune system stimulation by improving antigen presentation (depot formulations, delivery systems) and/or delivering co-stimulatory signals (Lockhart, 2003).

The immune system of the body works to defend the body against infections and microorganisms. If an animal is to respond correctly to a vaccine, it must have a functioning immune system. However, an immunocompromised animal can’t respond to the vaccine. The age of an animal can also influence vaccine responses. It's also been argued that being older slows down the immune system's reaction to vaccines (Shaw *et al*., 2010). Similarly, a poor diet might reduce nutrient availability for cell division and protein synthesis, which can suppress immunological responses (James, 2007). Generally, target host species including age, choice of vaccine approach (type), choice of immune response to be targeted, adjuvants, route of vaccination and vaccine dose are elements that have to be considered when developing veterinary vaccines.

Veterinary vaccine storage

Vaccines are sensitive biological substances that can lose their potency and efficiency if they are exposed to temperatures (heat and/or cold) outside of the optimal temperature range of +2 to $+8^0$C, or if they are exposed to sunlight. If the temperature is too high, the vaccine microorganisms or their antigenic structures may be damaged. Immunization programs are one of the most cost-effective methods of disease prevention. When planning or implementing immunization protocols, proper transportation, storage, and handling of vaccines are sometimes disregarded (Pastoret, 1999).

7.7.5. Fowl cholera vaccines

Vaccination is used as a preventive measure in many countries around the world to lower disease incidence. Several scientists advised that a local strain with a greater immunogenic value be used as the vaccine strain for bacterin production to control poultry cholera (Ievy *et al*, 2013). FC can be caused by any of the 16 Heddleston serotypes of *P. multocida*, though some seem to be more frequently related to the disease. Inactivated *P. multocida* vaccines with aluminium hydroxide or oil adjuvant are currently in use and are made from cells of serotypes chosen based on epidemiological data. Serotypes 1, 3, and 4 are frequently included in commercial vaccinations (OIE, 2015).

Live vaccines of low pathogenicity strains and inactivated vaccines are two types of vaccines that are commercially accessible. To reduce the incidence of FC, various immunization programs are used, particularly in broiler breeders and turkeys (Rimler *et al*., 1997). Bacterin is usually given as an intramuscular injection into the leg or breast muscles, or as a subcutaneous injection into the back of the neck. Two dosages are usually given every two to four weeks. Full immunity is not predicted until around 2 weeks after the second dose of a primary immunization series, as it is with most killed vaccines. Live vaccines, unlike inactivated vaccines, are usually given through drinking water. Vaccination of sick or underweight birds should be avoided since an adequate immune response may not be established in these situations (OIE, 2015).

3. MATERIAL AND METHODS

3.1. Experimental site

The study was undertaken at the Research and Development Laboratory of NVI, Bishoftu, Ethiopia. Bishoftu is located in Oromia Regional State, 45 kilometers southeast of Addis Ababa at 9°N and 40°E. The area has a bimodal rainy season, with mean annual rainfall ranging from 450 mm to 1,000 mm and temperatures ranging from 17°C to 30°C. The long season runs from late June to late September, and the short season is from February to April (http://www.mwud.gov.et/web/bishoftu/home)

3.2. Experimental animals

A total of 175 layer chickens (8 weeks old Bovans Brown) were hatched and grown in the experimental room of the Research and Development Laboratory, NVI to determine the shelf life of the FC vaccines. The chickens were checked for antibodies to FC by I-ELISA test at the start of the experiment and those found seronegative were recruited for the study. The chickens used in the studies were kept in a controlled environment in an isolated experimental facility. Before the chickens were brought to experimental rooms, the experiment rooms were disinfected and fumigated with formalin. During the experiment, the chickens were kept with free access to feed and water.

3.3. Experimental study design

This research was carried out from December 2019 to June 2022. The FC vaccine used for the study was manufactured from local seed strain *Avian P. multocida biotype A*, according to NVI's standard operating procedure (NVI/SOP, 2017), and the specific batch was chosen randomly for this study. The vaccine's shelf life was determined for 24 months following the immune response after two immunizations (primary and secondary) (booster) 3 weeks apart using formalin-inactivated alum adjuvanted FC vaccine stored at 2 - 8^0C. Chickens (25 chickens in each group) designated as groups 1, 2, 3, 4, 5, 6 and 7 received $1.65x10^9$CFU/ml or 0.5ml/dose of formalin-inactivated alum adjuvanted FC vaccine, through the IM route that was kept at 2-8^0C for 0.5, 3, 6, 9, 12, 18 and 24 months respectively.

3.4. Vaccine preparation

3.4.1. Inoculums and production media preparation

For the preparation of inoculums media, Tryptose soy broth (TSB) (HIMEDIA, M177-500G, Mumbai, India) was prepared as manufacturer instruction and supplemented with 10% filtered and sterile horse serum. FC vaccine production media was prepared according to the vaccine production media preparation procedure at Media Preparation Laboratory, NVI (NVI/SOP, 2017). This synthetic production media contents such as Peptone, Di Sodium hydrogen phosphate (Na_2HPO_4), Sodium chloride, Potassium dihydrogen phosphate (KH_2PO_4), Magnesium sulfate ($MgSO_4$), yeast extract and distilled water were mixed and dissolved and (pH adjusted to7.6 by HCL and NaOH, as required) and sterilized by autoclaving. Sterilization of glucose and serum was done by pressure filter with the pore size of 0.22µm in the Biosafety cabinet class II. After sterilization pre filtered and sterility checked glucose and serum were added to sterile production media. Both inoculums and production media were incubated at 37^0C for 72 hours before use to confirm its sterility.

3.4.2. Inactivated fowl cholera vaccine production

Formalin inactivated and alum adjuvanted FC vaccine production was performed according to the NVI Standard operative procedure of FC vaccine production procedure (NVI/SOP, 2017). Working seeds of Avian *P. multocida biotype A* (MK802880, NVI) were used for vaccine production. Lyophilized seed of *P. multocida biotype A* was diluted with 2 ml TSB homogenized thoroughly and then inoculated into sterile Tryptose soy agar (TSA) (SIGMA, 22091-500G, India) supplemented with 10% horse serum and incubated overnight at 37°C. The colonies were examined visually and microscopically after gram staining for their purity and cellular morphology, bipolar-staining short bacilli (cocco-bacilli) organisms and gram-negative. A single colony was transferred to a 2 ml hemolytic tube containing TSB with 10% horse serum and incubated for 6-8hours at 37°C. Then 0.5ml of the broth culture was transferred into 30 ml TSB supplemented with 10% horse serum and incubated overnight.

The purity of the *P. multocida type A* (PA inoculums) was checked by gram stain and 7ml of inoculums were inoculated into 300 ml of *P. multocida* PA production media supplemented with sterile 30 ml horse serum and 7 ml of glucose that was incubated for 24 hours with slow agitation at 80 rpm. Bacterial growth was checked by inspecting turbidity and measuring the pH of the culture. The pH of the culture was between 5.2-5.8, which is the optimum pH value for inactivation and corresponds to the desired titer of ($\sim\geq 10^8$ CFU/ml) as determined by the plate count method.

P. multocida suspension produced with optimum growth was inactivated by 1.5mlof 37% formaldehyde solution in the ratio of 1ml: 200ml and incubated at 37^0C for six days with slow agitation at 80 rpm to complete inactivation. The inactivated cultures were checked for sterility tests by culturing on media such as TSA, TSB, liver and meat broth and Sabouraud dextrose agar (SIGMA, 84088-500G, India). All inoculated and uninoculated negative control media were incubated at 37ºC for 48 hours for any bacterial contaminants except Sabouraud agar which was incubated at room temperature for two weeks to check fungal contaminants' growth (OIE, 2015).

The pH of inactivated cultures was adjusted to 7.0±0.3by using NaOH and then adjuvanted by addition of 60ml of 10% solution of Aluminium potassium sulphate (alum, $AlK(SO_4)_2.12H_2O$) and 240ml of saline water to 300 ml of inactivated culture in the ratio of 1:4:5 (i.e. 1 litre of alum: 4 litres of saline water: 5litre of inactivated culture). The mixture is continuously agitated using magnetic a stirrer before dispensing into 50ml sterile polypropylene vials. 2ml of this vaccine sample was also submitted to Molecular Biology Laboratory for identity test of *P. multocida type A* and *Mycoplasma* contaminants. The safety of the inactivated vaccine was checked in two rabbits by inoculating 1ml of the vaccine intramuscularly and observed for 14 days for any adverse reaction (OIE, 2015).

3.5. Vaccination and sampling

The inactivated alum adjuvanted FC vaccine was given intramuscularly (IM) to the breast muscle of chickens at a dose of 0.5ml per chicken. The original immunization was given on day zero, followed by a booster on day 21 after the initial vaccination (OIE, 2018). Blood was collected from each chicken from the wing vein just before primary

immunization (day zero), at days 21 and 35 after primary vaccination to determine serum antibody levels and the collected blood was allowed to clot at room temperature overnight for serum separation, and the separated sera were maintained at -20^{0}C for 16 - 25 days until I-ELISA test was performed.

3.6. Evaluation of humoral immunity by I-ELISA

Using the ID vet ELISA Kit, the relative amount of *P. multocida* antibodies in chicken serum was measured according to the manufacturer's recommendations (ID Vet Laboratories, France). The test was carried out on a 96-well ELISA plate that had been coated with *P. multocida* antigen. Frozen serum samples were thawed completely and mixed thoroughly before being diluted. Test sera were diluted five hundred fold (1:500) (i.e.5µl of each sample to be tested were added to 245µl of dilution buffer14 on serum dilution plate (uncoated), then10µl of the pre-diluted samples were added to 90µl of dilution buffer14 as many well as the sample quantity on *P. multocida* antigen-coated wells) while 100µl of undiluted negative and positive controls were also dispensed on the A1 B1 and C1 D1 coated wells, respectively. The plate was incubated for 30 min at 21^{0}C. So that antibodies specific to *P. multocida* form a complex with the coated antigens.

The plate was washed three times with approximately 300µl of wash solution (Phosphate-buffered saline, PBS) to remove any unbound material from the wells followed by the addition of a 100µl of anti-chicken horseradish peroxidase (HRP) conjugate to each well to form the antigen-antibody-conjugate-HRP complex. The plate was incubated for similar minutes and temperatures as before to enable binding to any attached chicken antibody in the wells. The unbound conjugate was washed away as above and 100µl of enzyme-substrate solution (Tetramethylbenzidine, TMB) was added for coloration depending on the number of specific antibodies present in the sample. The plate was covered and incubated for 15 min at 21^{0}C in the dark. In the end, 100µl of stop solution (sulphuric acid solution) was added to stop the reaction. The absorbance or optical density (OD) of the subsequent color development was measured by spectrophotometer at 450nm and the corresponding OD value was directly related to the amount of antibody to *P. multocida* present in the test sample. The antibody titer was determined from the OD values using the method stated in the test kit instruction.

Titre = antilog ($1.09(\log_{10}S/P)$) + 3.36

Where:
S/P = Mean OD of test sample – Mean OD of negative control
Mean OD of positive control – Mean OD of negative control

4.7. Calculation of the cut-off value

The ELISA cut-off value was obtained by determining the OD at a wavelength of 450 nm, and the value was calculated following the formula described in Kich *et al*., 2007. In the interpretation segment, an OD value of the chicken sera that was higher than the cut-off value was classified as seropositive and those less than the cut-off value were classified as seronegative.

Cut-off value (OD value) = Mean OD450 + 3 x standard deviations.

3.8. Ethical declaration

The chickens were kept in a secure, humane, and sanitary environment. Clean water and suitable food were provided to the chickens utilized in this investigation. All animal experiments were evaluated and authorized by the Animal Research Ethics Committee of the National Veterinary Institute laboratory animal ethics, Ethiopia, in accordance with internationally accepted guiding principles for animal experiment research.

3.9. Data analysis

Data were entered into Graph Pad Prism version 5 (Graph Pad Software Inc., USA) to test for significant differences in the assay methods used for the detection of FC antibodies. The Analysis of variance (ANOVA) test and Tukey multiple comparisons were used to determine the differences in mean antibody titers among immunized groups at different periods for FC vaccine shelf-life determination. Statistical significance was assumed at $p < 0.05$. For each experimental group, the mean OD value data was presented as an individual value. Mean and standard error of means were indicated in lines and error bars.

4. RESULTS

4.1. Comparison of antibody titre in chicken prior and post-vaccination of formalin-inactivated fowl cholera vaccine

The serum antibody response induced after each immunization was measured using an indirect ELISA as shown in Figure 1. Each group of chicken were seronegative with OD values below the cut-off value of 0.20 before being vaccinated. Non-significant difference (P>0.05) were observed in antibody titres among each group of chickens before immunization. The chicken serum antibody titre was significantly increased and higher than the cut-off value (Optical density (OD) = 0.20) in all the groups after 3 weeks of post-vaccination. Significant difference ($p < 0.0001$) was observed in antibody titre between prior and post vaccination among all experimental group throughout this investigation. The level of chicken serum antibody (IgG) titre was substantially increased after 2 weeks of the second (booster) immunization in each group of chickens vaccinated using the FC vaccine stored for different duration of time. The antibody titre of day 35 was significantly higher than day 21 in each group except group 3, 6 and 7 ($p > 0.05$).

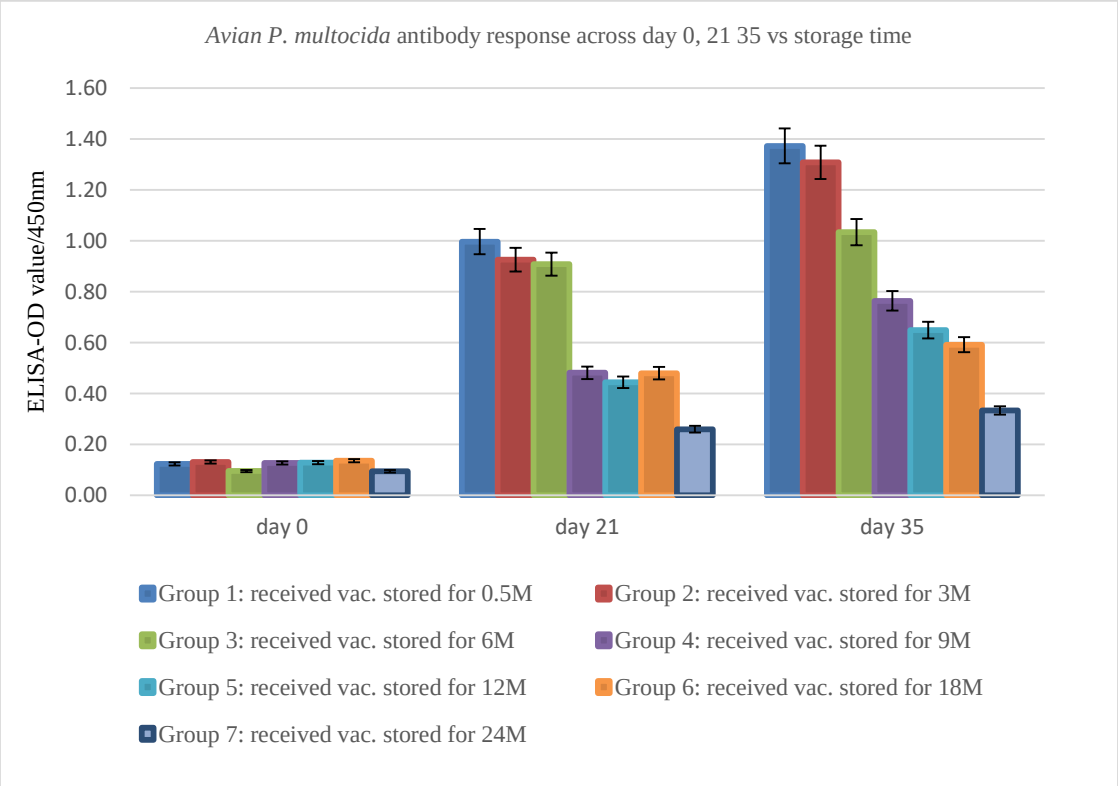

Figure 2: Sera antibody titer levels against *Avian P. multocida* among groups of immunized chickens with FC vaccine stored at 2 - 8^0C for different months

Group 1, 2, 3, 4, 5, 6, and 7 (n = 25 chicken each group) represented chicken groups that received vaccine stored for 0.5, 3, 6, 9, 12, 18 and 24 months respectively. The mean and standard error of means is indicated in lines and error bars. The mean OD value of the group was presented as data for each experimental group and used to compare the level of antibody titer among the groups.

A significant difference ($p > 0.05$) was not observed among group 1 (0.996±0.066), group 2 (0.926±0.075) and group 3 (0.908±0.072) in antibody titre and significantly higher than the remaining groups after primary immunization (day 21). Similarly, significant differences ($p > 0.05$) was not observed among group 4 (0.481±0.034), group 5 (0.444±0.046), group 6 (0.480±0.040) and group 7 (0.260±0.029) in antibody titre after primary immunization. However, there was significant difference ($p < 0.05$) among all groups except between group 1(1.372±0.110) and group 2 (1.308±0.084), group 18 (0.591±0.040) and group 24 (0.333±0.049) as well as among group 9 (0.764±0.043), group 12 (0.649±0.055), 18 (0.591±0.040) in antibody titre after booster immunization (day 35). Generally, the mean serum antibody titre was decreased from the first to the seventh group as the time of vaccine storage at 2-8^{0}C increased from two weeks to twenty four months. Although, the vaccine stored in recommended condition for up to eighteen months produced antibody response, under the same condition of storage for twenty four months produced the lowest antibody response.

5. DISCUSSIONS

For years, developing a vaccination to manage FC has proven to be a difficult task. All strains of *P. multocida* that will be used in a vaccine must be well-characterized, have a known serotype, be pure, safe, and immunogenic (OIE, 2018). An effective vaccine must be safe and needs to provide sustained protection with the elimination of the challenging infection. FC vaccines, both live and formalin-inactivated, have been widely utilized and have been successful in lowering infection and disease prevalence in chickens (Harper and Boyce, 2017). NVI has developed an effective and safe formalin-inactivated alum adjuvant FC vaccine that can be used countrywide to reduce the disease's impact in Ethiopia (Wubet *et al.*, 2019). The number of days that a product remains stable at the prescribed storage conditions, often known as the real-time shelf life was determined by this study.

This study was indicated that chickens in group 1 (4608.85± 317.80), group 2 (5378.38±302.56), group 3 (4863.38±234.17), group 4 (3144.01±153.74), group 5 (2645.10±228.39) were induced 100% protective antibody titres after secondary (booster) vaccination as reported by (*Wubet et al*., 2019; mean of serum antibody titre: 2472.97±603.47). However, immunized chickens in group 6 (2154.37±132.47) and group 7 (1855.07±253.89) were induced 95% protective antibody titre after secondary vaccination as compared to the mean serum antibody titre (1852.24±431.06) of the above challenge experiment report. Therefore, inactivated FC vaccine kept at 2 – 8^0C up to 24 months induced a better protective antibody response in chicken after secondary (booster) vaccination.

A stability testing is an important aspect of determining the quality and safety of vaccines that are subject to mandatory regulatory oversight. Stability study is used to ensure that vaccines have acceptable quality and, as a result, safety and efficacy profiles at the end of their shelf lives, or storage periods, when stored under the appropriate environmental conditions. Stability test is conducted to identify the storage period for intermediates, to determine or change a maximum shelf-life or minimum release standard for the final product, and to monitor vaccine stability following licensing requirements. Stability studies also provide a source of data for further comparability studies after manufacturing or formulation changes. Stability data ensures that a marketed product meets the criteria for the duration of its shelf life. Vaccine potency should be tested at regular times throughout

the date period to ensure vaccine stability. Vaccines are normally kept at 2 - 8°C and kept out of the deep freezer (-20^{0}C). Containers that have been partially used should be disposed of at the end of the day's operations (OIE, 2018).

In this work, the inactivated FC vaccine was prepared and stored at 2 - 8°C for up to 24 months, and the stability of the vaccine was tested by immunizing chickens before infection. The inactivated vaccine is usually given as an intramuscular injection into the leg or breast muscles, or as a subcutaneous injection into the back of the neck. Two doses are usually given at two to four week intervals. Full immunity is not predicted until around 2 weeks after the second dose of a primary immunization series, as it is with most killed vaccines. Vaccination of diseased or malnourished birds should be avoided since an adequate immune response may not be established in these situations (OIE, 2018). On day 0 and day 21, all chickens were vaccinated with a single dose and booster doses via IM, respectively. Antibody titres were determined using ELISA on serum samples obtained three times from each chicken before the single dose, three weeks after the single dose, and two weeks after the booster dose vaccination.

Each type of vaccination has its own set of stability concerns that must be taken into account during development. They cannot be stored in the same way as chemical products due to their instability. These stability issues affect the vaccine's safety and efficacy, and the loss of potency is irreversible. Light, heat, radiation, changes in the environment, and reactivity with the container or other components in the mixture can all induce vaccine instability. Even if ideal circumstances are maintained, potency may eventually decrease after manufacture. Maintaining proper storage conditions (e.g., 2–8°C) is critical for optimizing vaccine use (Dumpa *et al.*, 2019). A cold chain is usually required to maintain this temperature range. A cold chain is a temperature-controlled supply chain that is used to keep a specific temperature range. The cold chain approach is a widely utilized method for transporting vaccinations to different locations. Vaccines are commonly subjected to this technique to maintain appropriate temperatures and prevent the vaccine from losing effectiveness. An ideal cold chain maintains the temperature of the vaccine throughout the manufacturing, storage, and delivery stages (Dumpa *et al.*, 2019).

Currently, NVI recommends that inactivated FC vaccine have to be kept at 2–8°C until utilized for up to twelve months (i.e. its shelf life is twelve months). Moreover, this

vaccine is effective, safe and potent in this condition of storage up to date according to this recommendation. However, this study confirms that it induced an effective antibody response in chicken for up to twenty four months in the same storage condition as compared with previous vaccination-challenge experiment (Wubet *et al*., 2019). Unfortunately, because of the degradation of antigen, composition and other ingredients of the vaccine the antibody titre produced in chicken was decreased as the time of storage increased. However, no standard, report or evidence justifies the minimum protective antibody titre of this vaccine.

6. CONCLUSIONS AND RECOMMENDATIONS

The overall antibody titers to FC vaccine kept at 2–8°C for twenty four months were above cut-off values (seropositive) in this study. The mean antibody titer against *P. multocida* was significantly higher after two weeks, three and six months of storage. Moreover, antibody response was induced after nine, twelve, eighteen and twenty four months of storage. As a result, formalin-inactivated alum adjuvant FC vaccine kept at 2–8°C for twenty four months were induced antibody response.

Based on the above conclusion the following recommendations are forwarded:

- Formalin-inactivated FC vaccine could induce antibody response up to twenty four months of storage at 2–8°C.
- Determination of minimum protective antibody titer to be needed including challenge experiment to know the exact point of minimum protective antibody titter and to suggest its effectiveness for use in immunization of chicken against *P. multocida.*
- Different batches of vaccines need to be repeated to understand the consistency of the results.
- A thermostable vaccine that can be stored at room temperature, which is also ideal under field conditions, need to be formulated using sugars and other techniques.

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