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**ON-STATION AND FIELD EVALUATION OF INACTIVATED FOWL
CHOLERA VACCINE PRODUCED FROM LOCAL *Pasteurella multocida*
ISOLATES, ETHIOPIA**

MVSc Thesis



BY

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**ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND
AGRICULTURE, DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND
VETERINARY PUBLIC HEALTH**

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BISHOFTU, ETHIOPIA

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CHOLERA VACCINE PRODUCED FROM LOCAL *Pasteurella multocida*
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A Thesis submitted to the college of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements of the degree of Masters of Veterinary Science in Veterinary Microbiology

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June, 2018
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Title: **On-station and field evaluation of inactivated fowl cholera vaccine produced from local *Pasteurella multocida* isolates, Ethiopia**

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I declare that this thesis is my *real* work and that all sources of material used for this thesis have been appropriately acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowing under rules of the Library. I seriously declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

ALUM	Aluminum Potassium Sulphate
ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
CSA	Central Statistical Authority
CSY	Casein Sucrose Yeast
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ELISA	Enzyme Linked Immunosorbent Assay
FC	Fowl Cholera
IHA	Indirect Hemagglutination
IM	Intra Muscular
NMSA	National Meteorological Services Agency
NVI	National Veterinary Institute
OD	Optical Density
OIE	Office International Des Epizooties
PCR	Polymerase Chain Reaction
PI	Protective Index
PLC	Private Limited Company
RPM	Revolution per Minute
SOP	Standard Operating Procedure
TSA	Tryptose Agar
TSB	Tryptose Broth

ABSTRACT

Vaccination is one of the common preventive measures of fowl cholera. An experimental study on-station and field evaluation was carried out from November 2017 to April 2018. The objectives of this study were to evaluate the safety, immunogenicity and protective efficacy of inactivated fowl cholera vaccine recently developed at National Veterinary Institute of Ethiopia using local *Pasteurella multocida* isolate. The vaccine was produced with titer of 2.5×10^8 cfu/ml and evaluated in 8 weeks old chickens (n=60) by injecting 1 ml through IM (Group I; n=20), 0.5 ml IM route (Group II; n=20) and 20 chickens kept as unvaccinated control. The sera were collected from all groups' at day 0, 14, 21, 28, and 35 and subjected for antibody titer determination by indirect haemagglutination test. Boostered with the same dose and route after 21 days of primary vaccination. Finally, the vaccinated and control chickens were challenged using virulent strains of *Pasteurella multocida* at day 15 of post booster vaccination. The study at field level was conducted using 200 chickens isolated in a separate compartment. The chickens were vaccinated with 0.5ml through IM route at day 0 and booster dose was given with 0.5ml after 21 day of primary vaccination. Sera were collected at day 0, 21 and 35 and examined by indirect haemagglutination test. Mean antibody titers of Group I were 1.6 ± 1.2 , 211.3 ± 2.1 , 244.5 ± 1.2 , 319.8 ± 1.2 and 502 ± 1.2 at day 0, 14, 21, 28 and 35, respectively. In Group II, mean antibody titers were 1.3 ± 1.2 , 203.7 ± 3.0 , 234.2 ± 1.2 , $367.2 \pm 1.2.5$ and 452.9 ± 1.2 at day 0, 14, 21, 28 and 35, respectively. In Group III, mean antibody titer remain constant. In case of protective efficacy, both 1 and 0.5ml dose had 87.5% protection. At field study, the mean antibody titers were 4.2 ± 1.1 , 310.4 ± 1.0 and 532.6 ± 1.0 at day 0, 21 and 35, respectively. Formalin killed fowl cholera vaccine induces immune response and mean antibody titer increases after booster vaccination in both control and farm condition. The protective efficacy and immunogenicity between 1 and 0.5 ml dose of the vaccine was found insignificant difference. Therefore, the use of 0.5ml dose with booster vaccination at day 21 after primary vaccination is recommended.

Keyword: *Chickens, immunogenicity, inactivated fowl cholera vaccine, Pasteurella multocid*

1. INTRODUCTION

The poultry sector continues to grow and industrialize in many parts of the world. An increasing population, greater purchasing power and urbanization have been strong drivers of growth (FAO, 2014). The total numbers of chickens in Ethiopia were 56 Million and now reached to be 65.87 Million with expected increase in the future (CSA, 2011, Abdo *et al.*, 2016). Poultry production is an important economic activity in Ethiopia. It occupies a unique position in terms of high quality protein food contribution to rural smallholder farming families in Africa and particularly in Ethiopia (Tadelle and Ogle, 2001). Despite the fact that poultry industry is growing fast in Ethiopia, the industry is facing many constraints among which infectious diseases are the predominant ones with significant impact on the development of poultry industry (Dana *et al.*, 2008).

Fowl cholera is one of contagious bacterial disease of domesticated and wild avian species caused by infection with *Pasteurella multocida*. It is typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality in older birds. Chronic infections also occur with clinical signs and lesions related to localized infections. The pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection. Common synonyms for fowl cholera are avian pasteurellosis and avian hemorrhagic septicaemia (OIE, 2015). This disease has been reported as an important disease in domestic poultry for more than 200 years that causes devastating economic losses to poultry industry worldwide (Aye *et al.*, 2001). In poultry, it is often associated with severe economic crisis due to poultry production loss (Marza *et al.*, 2015), in which the acute form of fowl cholera is associated with high mortality, resulting in a significant economic impact on the poultry industry, while chronic infections and asymptomatic carriers result in the persistence of bacteria within flock. The bacterium can be disseminated within a flock and between houses by secretion and excretion that contaminate the environment and diagnosed clinically by excretion from mouth, nose and ears, and cyanosis of comb and wattles (Glisson *et al.*, 2013).

Vaccination is considered as one of the common preventive measures worldwide to reduce the prevalence and incidence of disease (Kardos and Kiss 2005). Many authors explained that the widespread distribution of many diseases had negative impact on the chicken production performance in developing countries and found to alert the timely vaccination strategies of quarantine (Sharman, 2010). Both live and inactivated vaccines have been attempted to control the disease (Glisson *et al.*, 2008). The live attenuated vaccines give good protection, having a long duration of immunity and cross-protection against different serotypes of *Pasteurella multocida*, it is less implemented in many countries due to the side effects, reactions including the localization of the organisms in the joints and sometimes causing lung infection and the principal one is the lack of regular maintainable attenuation or its instability and as a result, there is a risk of regaining its virulence, where as formalin inactivated vaccines are the best to protect animals without side effects having more advantages over the attenuated live vaccines (OIE, 2004). Immune responses vary according to breed, age and rearing zone (Rana *et al.*, 2010), in such a way that the immune response due to age variation has been observed greatly in younger chickens (1-5 weeks of age) and birds vaccinated at 1 or 2 weeks of age appear to be consistent with the relatively low humoral antibody response (Dick and Avakian 1991). Efficacy of a vaccine depends on many factors including the immunogenic characteristic of the vaccine strain, and it is widely accepted that a local strain having immunogenic value should be selected as the ideal vaccine strain to prepare effective vaccine to control a particular disease like fowl cholera (Akhtar *et al.*, 2016)

All veterinary vaccines administered to animals should be tested for safety and, if possible, for efficacy in the field, before being authorized for general use. Field studies are designed to demonstrate efficacy under working conditions and to detect unexpected reactions, including mortality that may not have been observed during the development of the product. Under field conditions there are many uncontrollable variables that make it difficult to obtain good efficacy data, but demonstration of safety is more reliable (OIE, 2018). Even though inactivated fowl cholera vaccines have been produced in other countries, in Ethiopia the poultry enterprises have experienced problems with the disease due to unavailability of the vaccine. To overcome the problem, vaccine were imported

from others country. The absence of vaccine has created the need of fowl cholera vaccine to be produced from local strain to reduce further economic losses to the poultry industry. The availability of an effective locally produced vaccine will reduce the dependence on imported vaccine, foreign exchange of the country and maximize the vaccine production per year. To alleviate this problem there has been a strong effort to develop the vaccine at National Veterinary Institute, Ethiopia. NVI is the only institute which produced veterinary vaccine in Ethiopia but so far fowl cholera vaccine is not produced. Therefore, large scale production of inactivated fowl cholera vaccine is vital to control fowl cholera in Ethiopia, hence, achieve the planned chicken production in the country. To this end, inactivated fowl cholera vaccine using three different adjuvants (alum, oil and aluminum gel) has been developed awaiting large scale production (Wubet, 2017) and subsequent quality control evaluation both at station and field levels with respect to safety, immunogenicity and protective efficacy.

Therefore, the objectives of the present study were:

- ✓ To evaluate inactivated fowl cholera vaccine of its safety and immunogenicity (seroconversion) both at on-station and field level.
- ✓ To compare 1 and 0.5ml dose of inactivated fowl cholera vaccine.
- ✓ To determine protective efficacy of inactivated fowl cholera vaccine through experimental challenge in target hosts.

2. LITERATURE REVIEW

2.1. Back ground

Fowl cholera has been recognized as a disease of poultry for over 200 years. About 100 years ago, Pasteur isolated the organism and used it in one of the first vaccine. In the United States, *P.multocida* is an important worldwide primary and opportunistic pathogen as well as the common commensal of many wild and domesticated animals (Ekundayo *et al.*, 2008). Christensen and Bisgaard,(2000) state that no country can be considered free from fowl cholera, because *P. multocida* has a broad habitat, including the mucosal surfaces of a wide range of domestic and wild birds and mammals. Further, they state that processed poultry products are not considered to present a major risk of infection transmission, due to the delicate nature of *P. multocida*. It would be difficult to justify the imposition of sanitary measures against *P. multocida* on imported poultry meat.

2.2. Etiology

Pasteurella multocida, the causal agent, is a small, gram-negative, non motile rod with a capsule that may exhibit pleomorphism after repeated subculture. *P.multocida* is considered a single species although, it includes three subspecies: *pasteurella multocida*, *pasteurella septica*, and *pasteurella gallicida*. Subspecies *Pasteurella multocida* is the most common cause of disease, but *pasteurella septic* and *pasteurella gallicida* may also cause cholera like disease (Jens, 2016). The relationship between subspecies and serovars of *P.multocida* obtained by published serotyping systems has not been elucidated. For many years, passive haemagglutination tests have formed the basis for a serogrouping system based on specific capsule antigens whereas tube agglutination and gel diffusion precipitin tests have been used to detect somatic antigens (OIE, 2015). Antigenic characterisation of *P. multocida* is accomplished by capsular serogrouping and

somatic serotyping. Capsular serogroups are determined by a passive haemagglutination test (Carter, 1972). *Pasteurella multocida* strains are classified into serogroups A, B, D, E and F based on capsular antigens, and further classified into 16 serotypes (1 to 16) primarily based on lip polysaccharide antigens (OIE, 2008; Wage *et al.*, 2013). A non serological disk diffusion test that uses specific mucopolysaccharidase to differentiate serogroups A, D, and F have been developed (Rimler, 1994). A specific multiplex capsular PCR assay has been developed that allows for rapid and specific capsular typing (Townsend *et al.*, 2001). Fowl cholera is caused by *P. multocida* type A: 1, A: 3 and type D in Asian countries (Rajeev *et al.*, 2011). Among the different serogroups, serotype A:1 strains causes 80% mortality, in contrast 20% mortality caused by type D strains of fowl cholera in chickens (Mohamed *et al.*, 2012).

2.2.1. Biology of *Pasteurella multocida*

Pasteurella multocida is aerobic and facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished using media such as blood agar, trypticase soy agar or dextrose starch agar, and isolation may be improved by supplementing these media with 5% heat-inactivated serum. Maintenance media usually do not require supplemental serum. Colonies range from 1 to 3 mm in diameter after 18–24 hours of incubation. They usually are discrete, circular, convex, translucent, and butyraceous. Capsulated organisms usually produce larger colonies than those of non capsulated organisms (OIE, 2015). *Pasteurella multocida* is a fairly delicate organism which is easily inactivated by common disinfectants, sunlight, drying or heat, and experiments suggest that *P. multocida* will survive for a maximum of thirty days in the environment (Rimler and Glisson, 1997). Consequently, contaminated environments are not thought to serve as reservoirs for periods of more than thirty days (Christensen and Bisgaard, 2000).

2.2.2 Antigenic type

Strains of capsular serogroups A and somatic serotypes 1, 3 and 4 are recognized as the causative agent of fowl cholera (Glisson *et al.*, 2013). The ability of *P. multocida* to invade and multiply within the host is enhanced by the presence of its capsule, a polysaccharide structure that is one of the most important virulence factors for this species (Willkie *et al.*, 2012). Functions assigned to the capsule include desiccation resistance, antiphagocytic activity and interaction with the complement system (Boyce *et al.*, 2000).

Pathogenesis of *P. multocida* is a complex interaction between host specific factors and specific bacterial virulence factor; therefore, understanding the disease pathogenesis is complex and depends on the bacterial strain, the animal model and their interactions (Harper *et al.*, 2006). Capsular serotype A and D produce dermonecrotic toxin encoded by *toxA* gene (Glisson, 2008; Yap *et al.*, 2013). Capsule and lip polysaccharide, fimbriae, dermonecrotic toxin, hemoglobin binding protein and outer membrane proteins are the key virulence factors (VF) for *P. multocida* (Adler *et al.*, 1999; Fuller *et al.*, 2000; Ewers *et al.*, 2006).

Host invasion, colonization, and tissue injury are facilitated by these virulence factors (Hunt *et al.*, 2000; Harper *et al.*, 2006). Therefore, to prevent and control of *P. multocida* infection requires information and knowledge about virulence factor. Besides, there was correlation between virulence factor and capsular serogroups, hemoglobin binding protein (encoded by *hgbA*) and the dermonecrotic toxin (encoded by *toxAgene*) associated with serogroups A, B, and D, respectively (Ewers *et al.*, 2006).

2.2.3. Taxonomy

The taxonomy of the family *Pasteurellaceae* Pohl 1981 appears to be as complex as that of *Enterobacteriaceae*. 16S rRNA sequencing indicates that the family should be divided into more than 20 genera. According to phylogenetic investigations, the genus

Pasteurella sensu stricto includes three sub clusters, two of which represent taxa mainly associated with avian hosts. True species of the genera *Actinobacillus* and *Haemophilus* have not been reported from birds. Several new taxa, which have been shown to belong to the family *Pasteurellaceae* Pohl 1981, have been reported from birds. Some of these seem to represent genus-like structures. Due to a high degree of host-specificity observed for many taxa belonging to the family, the existence of many more species can be foreseen as more avian species are examined (Christensen and Bisgaard, 1997). *P. multocida* is a Gram-negative, non-motile, coccobacillus, capsulated, non-spore forming bacterium occurring singly, in pairs or occasionally as chains or filaments belonging to the *Pasteurellaceae* family and its colony usually appears grey and viscous with strong mucinous odor (Ashraf *et al.*, 2011; Ievy *et al.*, 2013; Akhtar, 2013).

2.3. Epidemiology

2.3.1. Distribution

P. multocida possesses a worldwide distribution and causes respiratory and septicemic disease in more than 180 species of birds (Samuel *et al.*, 2007). Infection causes severe, systemic disease that is invariably fatal and highly contagious, affecting both domestic poultry and wild birds (Carver *et al.*, 2013).

2.3.2. Host range

Fowl cholera occurs throughout the country wherever poultry is produced, having extensive host range including chickens, turkeys, ducks, waterfowl, geese, pheasants, pigeons, sparrows, and other free-flying birds. The wide range of avian hosts in which fowl cholera has been reported suggests that all types of birds are susceptible. Among types of poultry, turkeys are most affected. Most or all in an infected flock may die within a few days. The disease usually occurs in young mature turkeys, but all ages are highly susceptible (Glisson, 2008).

2.3.3. Transmission

The presence of feather shafts in the ventricular lumen of the majority of larid carcasses diagnosed with avian cholera suggests scavenging of birds that died from avian cholera as a major mode of transmission (Michelle *et al.*, 2016). Monthly mean temperature, relative humidity and rainfall with 2-3 months lag, can be correlated with avian cholera incidence. The final model had good predictive ability for the occurrence of avian cholera and climate variability plays an important role in avian cholera transmission in Guangxi province (Qin *et al.*, 2017).

The organism may enter the body either through the digestive tract or the respiratory system, the important site of infection believed to be the respiratory tract. Rapid bird-to-bird spread is relatively easily accomplished. Carrier birds seem to play a major role in the transmission of disease. The potent sources of infection are the excretions of visibly sick or apparently healthy carriers and surviving birds from diseased flocks. The excretions from the mouth, nose and conjunctiva help in rapid dissemination of the organism through contamination of soil, water, feed, etc. Improper disposal of carcasses of infected birds can contaminate water supplies such as surface tanks, ponds, lakes, and streams and leads to sporadic outbreaks in the poultry farms. Mechanical transmission occurs by farm workers, contaminated shoes or equipment. Animals other than birds, such as dogs, cats, pigs and other farm animals/mammals may serve as reservoirs of infection and actively spread the disease. Wild birds, rats, and flies can also potentially carry the organism into a flock. Environmental stress inducers like improper and unhygienic housing, ventilation, cold and wet climates help in the transmission of the disease and flock outbreaks. The disease is not transmitted vertically through the egg (Glisson, 2008).

2.4. Diagnostic methods of fowl cholera

Presumptive diagnosis may be based on the observance of typical signs and lesions and/or on the microscopic demonstration of bacteria showing bipolar staining in smears of tissues, such as blood, liver, or spleen. Mild or chronic forms of the disease also occur where the disease is endemic, with localized infection primarily of the respiratory and skeletal systems (OIE, 2015).

2.4.1. Clinical Signs

Clinical signs of acute fowl cholera are inappetence, fever, ruffled feathers, oral mucus discharges, dyspnea and watery or yellowish diarrhoea (Rhoades and Rimler, 1990). Birds suffering from chronic form of the disease may show depression, conjunctivitis, dyspnea, lameness, torticollis, swelling of the wattles, sinuses, limbjoints, footpads and sternal bursae (Christensen and Bisgaard, 2000). In cases with significant pulmonary involvement, there will be loud respiratory rales and coughing as the disease progresses. Depending on the particular strain of *P. multocida* involved, there may be high to very high morbidity and mortality. With less virulent strains, some affected bird may slowly recover, after a variable period of depression. With more virulent strains, death usually occurs quickly after a brief period of prostration, accompanied by convulsive wing flapping and paddling. Birds which survive the acute disease may recover completely, or may develop an exudative arthritis in leg or wing joints. Arthritis may occur without signs of acute systemic illness, particularly in very young or old birds (Willkie, *et al.*, 2012).

2.4.2. Pathology

Chronic infections also occur with clinical signs and lesions related to localized infections. The pulmonary system and tissues associated with the musculoskeletal system are often the seats of chronic infection (OIE, 2008). The most common gross necropsy findings in the birds with confirmed avian cholera were acute fibrinous and necrotizing

lesions affecting the spleen, air sacs, and pericardium, and nonspecific hepatomegaly and splenomegaly (Michelle *et al.*, 2016).

2.5. Detection method of fowl cholera

Identification and characterization of *P. multocida* has relied on the ability to cultivate or purify the organism in the laboratory. The purified organism is subsequently classified according to phenotypic traits such as morphology, carbohydrate fermentation patterns and serological properties. However, culture conditions can influence the expression of these attributes thus diminishing the stability and reliability of phenotypic methods for strain identification (Matsumoto and Strain, 1993; Jacques *et al.*, 1994).

Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that yield to the acute form of the disease, and from exudative lesions of birds with the chronic form of the disease, is generally easily accomplished. Isolation from those chronically affected birds that have no evidence of disease other than emaciation and lethargy is often difficult. In this condition or when host decomposition has occurred, bone marrow is the tissue of choice for isolation attempts. The surface of the tissue to be cultured is seared with a hot spatula and a specimen is obtained by inserting a sterile cotton swab, wire or plastic loop through the heat-sterilized surface. Alternatively the sterilized surface can be cut with sterile scissors or scalpel and the swab or loop inserted into the cut without touching the outer surface (OIE, 2015).

2.5.1. Morphology and cultural characteristics

Identification of *P. multocida* can be done based on morphological study, staining properties, cultural and biochemical characteristic, as described by Cheesbrough (2006) and Cultural and morphological examinations can be conducted as described by Cowan and Steel (2004). Accordingly, Samples suspected of fowl cholera are first seared with spatula and incised with a small sterile scalpel blade and forceps. The specimen is

inoculated directly into tryptose broth medium, incubated for 2–3 hours, transferred to Casein Sucrose Yeast (CSY) agar, blood agar, nutrient agar, MacConkey agar and citrate agar. Growth of the organism, size of colony, pigmentation and their ability to produce any change in the medium like haemolysis on blood agar can be examined. If our sample is swab from these organs, it is inoculated directly onto selective medium, such as Casein Sucrose Yeast (CSY) agar, blood agar and incubated aerobically at 37⁰C for 24 hours. Then, suspected colonies subjected to Gram's and methylene blue staining for cellular morphology. Gram stain result showing gram negative, with bipolar coccobacilli characteristics were considered as *P. multocida*.

2.5.2. Biochemical characteristics

Phenotypic characterization of *Pasteurella multocida* by biochemical reaction from various samples based on the basis of sugar fermentation reaction (Cowan and Steel, 2004). *Pasteurella multocida* does not cause haemolysis, it is not motile and only rarely grows on MacConkey agar. It produces catalase, oxidase, and ornithine decarboxylase, but does not produce urease, lysine decarboxylase, beta-galactosidase, or arginine dihydrolase. Phosphatase production is variable. Nitrate is reduced; indole and hydrogen sulphide are produced, and methyl red and Voges–Proskauer tests are negative (Glisson, *et al.*, 2008).

Table 1: Biochemical test result of *p. multocida*

Test type	Result
Hemolysis on blood agar	_*
Growth on MacConkey's agar	-
Indole production	+
Gelatin liquefaction	-
Catalase production	+
Urease production	-
Glucose fermentation	+
Lactose fermentation	-u
Sucrose fermentation	+
Maltose fermentation	-u
Ornithine decarboxylase	+

*Test reaction results: - = no reaction; + = reaction; -u = usually no reaction; (OIE, 2015).

2.5.3. Pathogenicity test

Pathogenicity test of strains of *P. multocida* can be carried out from pure colony grown for 18 h in a shaker-combincubator at 37°C in Brain Heart Infusion (BHI) broth. About 0.2 ml each culture containing approximately 2.4×10^8 colony forming units/ml can be inoculated into three test mice by the intraperitoneal and observe for 72 h to look at the mortality pattern. If the organism is re-isolated from heart blood collected from dead mice on a blood agar plate and an impression smear from the liver reveals the agent by Giemsa method of staining and again if the re-isolated colonies showed similar

characteristics of *P. multocida*, and impression smears revealed typical bipolarity of the organism, *P. multocida* is said to be pathogenic (Shivachandra *et al.*, 2005).

2.5.4. Serological identification

Serological tests, such as enzyme-linked immunosorbent assays (ELISA), agglutination and indirect hemagglutination assay (IHA) have been used to identify antibodies against *Pasteurella multocida* in poultry sera (Marshall *et al.*, 1981). Beside, indirect hemagglutination procedure can be developed for the identification of different capsular antigens of *Pasteurella multocida* (Solano *et al.*, 1983)

The immunogenicity is studied by the determination of the serum antibody titer by IHA and ELISA test suggested on (OIE, 2015). The potential use of IHA test as a practical method for determination of immunological response of poultry to vaccination programs has been evaluated previously (Islam *et al.*, 2017 and Amin *et al.*, 2013).

2.5.5. Polymerase chain reaction (PCR) techniques

In the recent years, the phenotypic differentiation tools have been frequently replaced with the genotypic methods (Taylor *et al.*, 2010). PCR-based typing techniques were found to be rapid and highly sensitive for identifying and differentiating the strains. Confirmation of the isolated organism as *P. multocida* can be done based on PCR targeting capsular gene cap specific for *P. multocida* as described in (OIE, 2008).

2.6. Prevention and control of fowl cholera

2.6.1. Vaccine and Vaccination of fowl cholera

Vaccination is practiced as preventive measures in many countries of the world to reduce the incidence of the disease. Various scientists suggested that a local strain of higher

immunogenic value should be selected as vaccine strain for preparation of bacterin with a view to control fowl cholera (Ievy *et al*, 2013). Fowl cholera may be caused by any of 16 Heddleston serotypes of *P. multocida*, although certain serotypes appear to be more often associated with disease. The *P. multocida* vaccines in general use are inactivated, containing aluminium hydroxide or oil adjuvant, prepared from cells of serotypes selected on the basis of epidemiological information. Commercial vaccines are usually composed of serotypes 1, 3, and 4 (OIE, 2015).

Broad category of vaccines are commercially available include live vaccines of low virulence strains and inactivated vaccines. Different vaccination programs are practiced to minimize the impact of fowl cholera, particularly in broiler breeders and turkeys (Rimler *et al.*, 1997). Bacterin is normally administered by intramuscular injection in the leg or breast muscles, or subcutaneously at the back of the neck. Two doses are typically administered at 2–4-week intervals. As with most killed vaccines, full immunity cannot be expected until approximately 2 weeks after the second dose of a primary vaccination course. Unlike that of inactivated vaccine, live vaccines are typically administered in the drinking water. Vaccination of diseased birds or those in poor nutritional status should be avoided as a satisfactory immune response may not be generated in such circumstances (OIE, 2015).

2.6.2. Method of manufacture

The manufacture of immunological veterinary medicinal products has special characteristics that should be considered when implementing and assessing the quality assurance system. Due to the large number of animal species and related pathogenic agents, the variety of products manufactured is very wide and the volume of manufacture is often low. The products should be protected against organic or inorganic contamination and cross-contamination. The ability of media to support the desired growth and effectiveness should be properly validated in advance and media should be first sterilized in situ or in line. One of the recommended sterilization of media is Steam heat under pressure (OIE, 2016). Production cultures of each bacterial isolate to be included in the

final product are prepared. The cultures are typically started in small vessels and sub passaged into progressively larger volumes of media until the desired production volume is achieved. Each production culture is inactivated by formalin or other acceptable means. All of the component cultures are mixed, and usually blended, with an adjuvant prior to filling sterile final containers (USDA, 2001; OIE, 2004)

2.6.3. Master seed management

A master seed should be established for each microorganism used in the production of a product to serve as the source of seed for inoculation of all production cultures. Records of the source of the master seed should be maintained and for each seed, the highest and lowest passage levels that may be used for production should be established and specified in the outline of production or standard operating procedure (SOP) based on marketing authorization data (OIE, 2016) All strains of *P. multocida* to be incorporated into a bacterin or vaccine must be well characterized, of known serotype, pure, safe and immunogenic. The culture(s) that is evaluated and characterized is designated by lot number and called a master seed. All cultures used in the production of licensed bacterins or vaccines must be derived from an approved master seed(s) and must be within an accepted number of passages from the master seed lot (OIE, 2015).

A. Validation as a vaccine

Fowl cholera vaccine can be validated by testing efficacy, safety, stability and duration of immunity. The efficacy of veterinary vaccines should be demonstrated by statistically valid vaccination–challenge studies in the host animal, using the most sensitive, usually the youngest, animals for which the product is to be recommended. Data should support the efficacy of the vaccine in each animal species by each vaccination regimen that is described in the product label recommendation, including studies on the onset of protection when claims for onset are made in the product labeling and for the duration of immunity (OIE, 2012). Birds used in efficacy studies should be immunologically naive to fowl cholera and at the minimum age recommended for product use. The lot of product

used to demonstrate efficacy should be produced from the highest allowable passage of master seed. For live avian *Pasteurella* vaccines in the USA, 20 vaccine and 10 controls are used in each efficacy trial. Birds are challenged not less than 14 days after vaccination and are observed for 10 days after challenge. A satisfactory test requires that at least eight of the controls die and at least 16 of vaccinated survive. The arithmetic mean count of colony-forming units in the lot of product that is used to demonstrate efficacy is used as the minimum standard (immunogenicity standard) for all subsequent production lots of vaccine. Safety must be tested in each animal species (chickens, turkeys, and ducks) for which the product is recommended and overdose studies are typically required for live vaccines. Each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. If unfavorable reactions are seen, this finding should be included in a risk assessment, and it may be appropriate to designate maximum permissible serial potency requirements. The acceptability of the shelf life of a vaccine is confirmed by testing the product for potency at the end of the approved shelf life. At least three lots of vaccine are tested and must pass established potency requirements, and then vaccines are stored at 2–7°C and protected from freezing. Formal duration of immunity studies is not typically required, although it is important to check the requirements of individual countries (OIE, 2015)

B. In-process controls

The purity of the cultures is determined at each stage of production prior to inactivation. This may be achieved by microscopic examination or by culture. Killed cultures are tested for completeness of inactivation. Analytical assays to determine the levels of formaldehyde or other preservatives are done on bulk vaccine and must be within specified limits. During manufacturing, production parameters must be tightly controlled to ensure that all batches are produced in the same manner as that used to produce the serials used for efficacy studies (OIE, 2018).

C. Batch control

In batch control of this vaccine test like sterility or purity, identity, safety and formaldehyde content should be checked for each batch. Sterility tests are done on filled vaccine and each lot must pass sterility requirements. The identity of the antigens in inactivated products is typically ensured through the master Seed concept and good manufacturing controls. Separate identity testing on completed product batches is not required in the USA, but procedures may differ in other countries. Safety testing is conducted on each bulk or filled vaccine lot and may be assessed in birds vaccinated for batch potency tests and Vaccines inactivated with formaldehyde are tested for residual formaldehyde (OIE, 2015).

2.6.4. Factors affecting vaccine effectiveness

Vaccine effectiveness can be defined as the ability of a vaccine to prevent specific outcomes in a “real life” situation. Assessing vaccine effectiveness is necessary in order to establish the actual benefit of the vaccination in the field. Vaccine effectiveness estimates may significantly differ from vaccine efficacy measured during vaccine trials. Demonstration of effectiveness may be derived from clinical endpoint efficacy studies. In some cases, immunogenicity data may be sufficient for a demonstration of effectiveness and clinical endpoint efficacy data are not required. Whether immunogenicity data provide sufficient evidence of effectiveness depends on the strength of evidence that the immune response endpoints predict clinical protection and whether there are sufficiently validated assays to measure those endpoints. In such cases, immunogenicity is considered a good proxy for vaccine efficacy (Orenstein, 1985).

A safe vaccine is not simply one that has been manufactured, tested and found to be safe in clinical trials. Important as those aspects are, there are other possibilities for making immunization safer. These include safe transport to the point of administration, safe administration, safe disposal of the vial and injection equipment and post-marketing

surveillance to detect any unexpected reactions as soon as possible (Clements *et al.*, 2004).

There are several factors within the control of the vaccinator or farmer that may affect vaccine efficacy. First, vaccines should be stored at the appropriate temperature recommended by the manufacturer and each vaccine has an expiry date printed on the vial which should follow. All animals do not respond equally well to vaccination and some may not mount an effective immune response due to some factors influencing vaccine like maternal Antibody, nutrition, immune system of animal and vaccine factors. Regarding maternal antibody, infants are born with immature immune systems, making it difficult for them to effectively respond to the infectious pathogens encountered shortly after birth. Maternal antibody is actively transported across the placenta and serves to provide protection to the newborn during the first weeks to months of life. However, maternal antibody has been shown repeatedly to inhibit the immune responses of young children to vaccines (Kathryn and Edwards 2015).

Veterinary vaccines whether attenuated or noninfectious from the different manufacturers can vary in their potency, efficacy and duration of immunity. Attenuated vaccines tend to induce stronger and long lasting immunity than non-infectious vaccine. Noninfectious vaccines include killed, toxoid; subunit and DNA vaccines are safer and more stable than attenuated vaccines. However, due to risk of using live vaccines in pregnant or immunosuppressed animals as well as the risks of shedding vaccine virus, non-infectious is preferred for some diseases (James, 2007).

Adjuvant is another vaccine factor that affects the effectiveness of vaccine. The innate immune system is the first line of immunological defense and is the arm of the immune response that is activated by adjuvant in vaccines. Therefore activation of this innate system by adjuvant is required for effective vaccination (Pulendran, 2011). All non-living vaccines require an adjuvant to provide an adequate immune response. A wide range of adjuvants are used in animal vaccine including aluminum salts and derivatives of the glycosides saponin. Adjuvant Agents that increase the stimulation of the immune system

by enhancing antigen presentation (depot formulation, delivery systems) and/or by providing co stimulation signals (Lockhart, 2003).

The immune system of the body functions to protect it against invasion by germs and microbes. When an individual is vaccinated against a disease or an infection, the immune system is prepared to fight the infection. An animal must have an effective immune system if it is to respond appropriately to a vaccine. An animal's age may affect vaccine responses. Old age has also been suggested to suppress vaccine response (Shaw *et al.*, 2010). Similarly, Poor nutrition can suppress immune responses by decreasing nutrient availability for cell division and protein synthesis (James, 2007).

2.7. Status of fowl cholera in Ethiopia

Poultry mortalities due to disease are estimated to range from 20% to 50% but they can rise as high 80% during epidemics in Ethiopia. It has been indicated that fowl cholera is among the major problems limiting chicken production in Ethiopia (Aberra and Tegegne 2007). According to Ethiopia animal health yearbook 2011, from the passive surveillance disease reporting, there were 6 outbreaks with 336 cases and 72 deaths with total population at risk estimated to be 108,775. Unfortunately, passive surveillance disease reports, to-date, in Ethiopia are not laboratory confirmed and are, rather, reports based on suspicions and on owner information. Ethiopia, even though the in frequent complaints of the state and private poultry farms due to the high morbidity, mortality, loss of production and high treatment cost pertaining this disease to the National Veterinary Institute, the prevalence of the disease has not been quantified (Bitew, 2008).

Most poultry outbreaks, particularly in more remote parts of the country, remain undiagnosed and dead chickens are simply discarded. Therefore, information on the prevalence and significance of infectious poultry diseases can only readily be obtained through indirect serological studies on apparently healthy chickens. It is difficult to design and implement chicken health development programs without an understanding of

the diseases present in the backyard poultry production system. One study revealed a high seroprevalence of fowl cholera (65%), and this constitutes the first report of fowl cholera seroprevalence in Ethiopia (Chaka *et al.*, 2012).

3. MATERIALS AND METHODS

3.1. Experimental site

Vaccine production and evaluation of the safety, immunogenicity and protective efficacy test on-station level was undertaken at the National Veterinary Institute (NVI), Bishoftu, Ethiopia. Field evaluation (safety and immunogenicity) of the vaccine was also conducted at poultry farm in Bishoftu owned by Addis Ababa University College of Veterinary Medicine and Agriculture. Bishoftu is located in Oromiya Regional State about 45 Km south east of Addis Ababa at an altitude of 1850 meter above sea level lying within geographic coordinates of 9°N latitude and 40°E and annual rain fall of 866 mm of which 84% is in the long rainy season June to September (NMSA, 2010). NVI is an organization responsible for producing quality vaccine which are based on research and development and involving in the control and eradication of live stock disease in the country (Figure 1).



Source: Google Map satellite

Figure 1: Map of the study location

3.2. Experimental animals

A total of 60 chickens (8 weeks old bovans brown) from Alema Poultry farm were purchased for on-station vaccine trial study. The chickens were screened and those free of antibodies against fowl cholera were included in the study. Chicken were fed with formulated feed supplied by Alema poultry feed processing PLC and potable tap water *ad- libtum*. The feed type used was starters feed, pullet's feed and layers feed which was changed according to age of chicken during the course of experiment.

For field evaluation of the vaccine, a total of 200 randomly selected layer chickens (bovans brown breed) from AAU-CVMA poultry farm were used. The chickens were isolated in a separate compartment though in same house from the rest of the flock for the purpose of the study. The farm has a total population of 3000 chickens and all (including those used for the study) were kept under same feeding and management system practiced in the farm.

3.3. Experimental Study design

The present research was conducted during the period of November 2017 to April 2018. The study was comprised two phases. The first phase was production of the first batch of fowl cholera vaccine according to the SOP developed during the trial vaccine development and evaluation of vaccine safety, immunogenicity and protective efficacy at NVI. The second phase was field evaluation of the vaccine at AAU-CVMA poultry farm. For on-station vaccine evaluation, a total of 60 chickens (8 weeks old bovans brown) were randomly classified into three groups (each group consists of 20 chickens). Group I and II were vaccinated and Group III unvaccinated control. Group I vaccinated with a dose of 1ml IM and Group II vaccinated with a dose of 0.5ml IM route. Identification of each chicken was done by wing tags.

The second phase field evaluation of the vaccine with regard to its safety and immunogenicity under farm condition. This was done at AAU-CVMA poultry farm on two hundred (200) randomly selected layer chickens maintained in an isolated compartment in same poultry house for follow-up and sera sampling. Bleeding, vaccination and challenge schedule were seen in Table 2.

Table 2 Schedule for vaccination, bleeding and challenge test

Group of chickens	Bleeding date	Dose	route	Primary vaccination	Secondary vaccination	Challenged with 1.67x10 ⁸ cfu/ ml
On-station						
Group- I	0,14,21,28, 35	1ml	IM	day 0	day 21	Day 35
Group-II	0,14,21,28, 35	0.5ml	IM	day 0	day 21	Day 35
Group-III	0,14,21,28, 35	Contr ol	IM	Not done	Not done	Day 35
Field study						
	0,21,35	0.5ml	IM	day 0	day 21	Not done

3.4. Vaccine preparation

Vaccine preparation was performed according to the (NVI/SOP, 2017). Chemicals (Annex2) except glucose and serum used for fowl cholera vaccine production media were mixed together and pH was measured using pH and adjusted at 7.4 then sterilize by autoclave. After sterilization pre filtered glucose and serum were added to production media. Sterilization of glucose and serum was done by pressure filter with the pore size

of 0.22 µm in the Biosafety cabinet class II. The total volumes of medium used for this vaccine production were 32lt.

3.4.1. Preparation of fowl cholera inoculums and production

For preparation of inoculums, lyophilized bacteria were diluted and allowed to grow in the tryptose agar plate. After 24 hours of incubation at 37 °C the clear colonies were grown on the agar plate. A single colony was transferred to 2ml of hemolytic tube containing TSB media then incubated for 7 hours at 37°C after which the purity was checked by gram staining. Then after, transferred to 1lt of fowl cholera inoculums media and incubated overnight.

The purity of fowl cholera inoculums was checked aseptically by gram's stain and inoculated into a bottle of fowl cholera production media then incubated for 24 hours with agitation of 80 rpm (NVI/SOP, 2017). Bacterial growth was checked by examination of smears, turbidity, and pH of the culture. The pH of culture were between 5.2-5.8. which is the optimum pH value for inactivation purpose.

3.4.2. Inactivation of the culture

Pasteurella multocida suspensions produced with optimum growth was inactivated by using 40% formaldehyde solution (final concentration of 0.5%) at 37°C and kept for 48 hours. Then anaculture placed at a temperature of 37°C for 72 hours to complete inactivation and further processed for sterility, inactivation and safety tests (Arshed, 2002; Bitew *et al.*, 2009).

3.4.3. Quality control of the anaculture

The anaculture was checked for its Purity and sterility by using Gram's stain and culturing on sterility test media such as tryptose agar, tryptose broth, VF broth and sabouraud agar media. All the test media was incubated at 37°C except sabouraud agar

which was incubated at room temperature. Un-inoculated media from each type was also incubated as a negative control. All these inoculated media was observed for seven days for any microbial growth. The safety of anaculture was done in laboratory animals. Three rabbits were injected with 1ml of inactivated culture intramuscularly from each bottle of anaculture, and observed for 14 days for any adverse reaction (OIE, 2015).

3.4.4. Adjuvation of vaccine

In this experiment Aluminum potassium sulphate (Alum) was used as an adjuvant and the ratio between the culture and the aluminum potassium sulphate was 10% (Bitew *et al.*, 2009). Then, the vaccine was dispensed into vials of 50ml volume capacity according to NVI Standard operative procedure (NVI/SOP, 2017). Finally, the quality control of inactivated fowl cholera vaccine was assessed by using the same method stated for anaculture.

3.5. Evaluation of inactivated fowl cholera vaccine safety, immunogenicity and protective efficacy at the NVI

3.5.1. Vaccination and sera sampling of experimental groups

In the station experiment, the chickens were acclimatized for a week at NVI experimental facility then vaccination of chicken was done intramuscularly of two groups each containing 20 chickens. Group I (vaccinated with 1ml) and group II (vaccinated with 0.5ml) while group III was left as non-vaccinated control. Primary vaccination was done at day zero followed by booster at day 21 after the primary vaccination (OIE, 2014). Blood was collected from all groups (vaccinated and non-vaccinated controls) by bleeding of the wing vein just before primary vaccination (day zero), at days 14, 21, 28 and 35 post primary vaccination for determination of serum antibody levels. All

experimental groups of chickens observed starting at day of vaccination and any deviation from normal health using observation of vital signs was recorded.

About two ml of blood without anticoagulant with 3ml syringe was collected from the wing vein of all experimental chickens and the syringes were held in slanted position and blood was allowed to clot at room temperature for an hour; clots were detached from the wall of the syringe by pressing the piston and were kept overnight at 4°C for separation of the serum. Then serum was carefully removed and centrifuged at 2000 rpm for 10 minutes and stored at -20 °C until analysis (Siddique *et al.*, 1997; Arshed, 2002; Akand *et al.*, 2004).

3.5.2. Determination of immune response

Sera samples were analyzed using indirect haemagglutination test described by Sawada *et al.*, (1982). Accordingly, Sera of the immunized and control birds were collected and tested by IHA. Briefly, 90 µl of PBS was first poured in the first row and 50 µl each well up to 10th well of vertical row of 96 well microstate plate. 10 µl of test serum was added in the 1st well and tenfold dilutions of serum ranging from 1: 10 to 1: 1280 were made by transferring 50 µl of the mixture from the 1st well to 2nd well and thus continuing successively up to the 8th well from where an excess amount of 50 µl of the mixture was poured off. A volume of 50 µl of capsular antigen sensitized sheep RBC was taken in each of the ten wells. The content of the wells of the test system was mixed by gentle agitation of the micro titer plate and kept at 37 °C temperature for 1 hour. Antigens are coupled to chemically modified erythrocytes that readily react with specific antibodies and results haemagglutination.

3.5.3. Challenge experiment

Fourteen days after booster vaccination, all vaccinated and control groups were challenged with live 1ml of virulent strain of *P. multocida* containing 1.67×10^8 cfu/ml (Annex 4) through intramuscular route according to previous studies by (Khan *et al.*,

1994; Islam *et al.*, 2004; Rahman *et al.*, 2004). Chicken were then followed for 14 days post challenge during which any clinical signs observed were recorded.

3.6. *P. multocida* isolation

Dead chickens were collected from experimental site of NVI for re- isolation of *P. multocida* from spleen, heart and liver. Re-isolation and identification of *P. multocida* was done based on morphological study, staining properties, cultural and biochemical characteristic (Annex 1 and 3), as described by Cheesbrough (2006). All samples were collected as per standard sample collection procedure by (Cowan 1985; Panna *et al.*, 2015). Confirmation of the re-isolated organism as *P. multocida* was done based on Polymerase Chain Reaction targeting capsular gene *cap* specific for *P. multocida* as (Annex5, 6, 7) described in OIE Manual (2008).

3.7. Evaluation of safety and immunogenicity of fowl cholera vaccine at field

3.7.1. Vaccination and sera sampling

In the field evaluation, in 200 birds randomly selected from AAU-CVMA farm were vaccinated with 0.5 ml of the vaccine intramuscularly at day zero and 21 (OIE, 2014). Blood was collected just before vaccination; day 21 and 35 post vaccination and determination of immune response were done by employing the same method stated for on-station.

3.7.2. Safety of fowl cholera vaccine at farm level

Evaluation of safety of the vaccine was done according to the OIE manual for vaccine safety parameter (OIE, 2015) vaccinated chickens were observed the whole day starting from time of vaccination up to 35 days on daily bases by professionals at the farm and

any deviation from normal health using observation of vital signs was recorded like depression, anorexia, ruffled feather and any reaction at the site of injection.

3.8. Ethical consideration

The Chickens were kept under safe, humane and hygienic conditions. Chickens used for this study were supplied with clean water, and appropriate feeds. All chickens were handled according to National veterinary institute laboratory animal ethics.

3.9. Data analysis

The data concerning, safety, immunogenicity and challenge tests was entered in to Microsoft Excel spreadsheet and coded for analysis using Stata version -12.0. Descriptive statistics such as proportions and averages were also used in summarizing quantitative data as required. The Analysis of Variance (ANOVA) was used to find out the differences in the mean antibody titers among immunized groups vaccinated with the different vaccine dosages and student t test was used to compare the antibody titer mean within the group. The desired level of precision and confidence level used for this study purpose was 5% and 95% respectively (Thrusfield, 2005). Analysis of protective efficacy data from the challenge experiment was evaluated by determining protective index (PI) as described by (Arshed, 2002).

$$\text{Protective Index} = \frac{\% \text{ mortality in control} - \% \text{ Mortality in vaccinated}}{\% \text{ Mortality in control}} \times 100$$

4. RESULTS

4.1. Safety test result of produced vaccine

Large scale production of fowl cholera vaccine using production medium resulted in a vaccine with a titer of 2.5×10^8 cfu/ml. Observation of all the chickens vaccinated with the inactivated fowl cholera vaccine showed no abnormal reaction to the vaccine like depression, anorexia, ruffled feather, and any reaction at the site of injection during the experimental time. In addition no morbidity or mortality was recorded, hence the vaccine was 100% safe in both on-station and field condition.

4.2. Immunogenicity of the inactivated fowl cholera vaccine

4.2.1. Immunogenicity at on-station study

Inactivated fowl cholera vaccine was administered at the dose rate of 1ml and 0.5ml with titer of 2.5×10^8 cfu/ml through intramuscular route in the group I and II chickens. The pre vaccination mean of IHA titers of sera samples of all vaccinated and control chicken were less than two. After the primary vaccination IHA antibody titers increased in both group. Booster vaccination (at day 21) resulted in rapid rise in IHA antibody titers in groups I and II. The mean IHA antibody titers in Group I were 1.6 ± 1.2 , 211.3 ± 2.1 , 244.5 ± 1.2 , 319.8 ± 1.2 and 502.3 ± 1.2 at day 0, 14, 21, 28 and 35 post vaccination respectively. Similarly, in group II, the mean IHA antibody titers at day 0, 14, 21, 28 and 35 post vaccination were 1.3 ± 1.2 , 203.7 ± 3.0 , 234.2 ± 1.2 , 367.2 ± 1.2 and 452.9 ± 12 respectively. However, in group III the IHA antibody titers remained constant (Figure 2).

Table 3: Geometric (Mean \pm SE) of IHA titer sera of chickens vaccinated with fowl cholera vaccine at different dose

Time	Group (vaccination)	Mean \pm SE	p-value
Day-0	Group I	1.6 \pm 1.2	0.687
	Group II	1.3 \pm 1.2	
	Group III	1.4 \pm 1.2	
Day-14	Group I	211.3 \pm 2.1 ^a	0.0001
	Group II	203.7 \pm 3.0 ^{ac}	
	Group III	1.41 \pm 2.0 ^b	
Day-21	Group I	244.5 \pm 1.2 ^a	0.0001
	Group II	234.2 \pm 1.2 ^{ac}	
	Group III	1.41 \pm 1.2 ^b	
Day-28	Group I	319.8 \pm 1.2 ^a	0.0001
	Group II	367.2 \pm 1.2 ^{ac}	
	Group III	1.41 \pm 1.2 ^b	
Day-35	Group I	502.3 \pm 1.2 ^a	0.0001
	Group II	452.9 \pm 1.2 ^{ac}	
	Group III	1.4 \pm 1.2 ^b	

The mean difference is significant < 0.05 level within Group for each day

^{abc} Means with different superscript letters within a column are different at (P<0.0001);

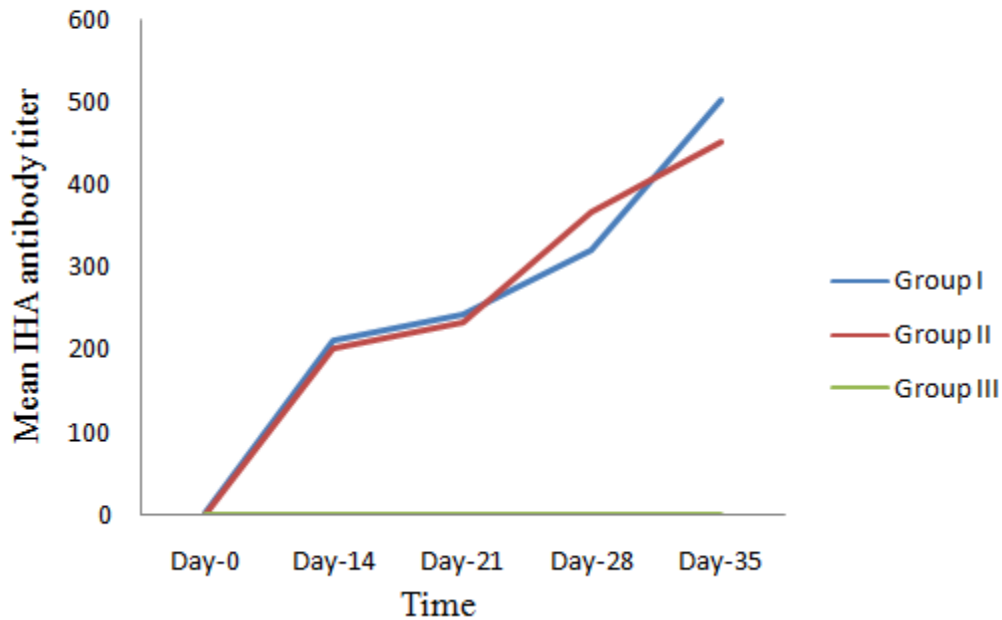


Figure 2: The comparison of serum mean IHA titer of chickens vaccinated and control and pre vaccination, post vaccination and post booster doses

4.2.2. Immunogenicity test result in field level

The mean IHA antibody titer is presented in the Table 4. The pre vaccination means of IHA titers of sera samples of all vaccinated chickens were 4.2 ± 1.1 . The primary vaccination improved IHA antibody titers mean to 310.4 ± 1.0 . Booster vaccination triggered the production of IHA antibody titers mean rapidly with 532.6 ± 1.0 . IHA antibody titer was significance increase when compare per vaccination and booster vaccination ($p < 0.0001$).

Table 4: Geometric mean and standard error of IHA antibody titer at farm level

Time	Mean± SE	p- value
Pre-vaccination(day 0)	4.2±1.1	
Day-21	310.4±1.0	0.0001 [®]
Day-35	532.6±1.0	

SE, standard error

[®] P<0.05, Significant difference among the means of antibody titers

4.3. Protective Efficacy Test Result

To measure the efficacy of the vaccine, all vaccinated and control chickens were challenged with virulent strain of *P. multocida* by intramuscular route at the dose 1.67×10^8 cfu/ml and the result is indicated in Table 5. After the challenge, the survival number and protection rate were counted. Out of 20 chickens 90% were survived and only two chickens were died in group I and also similar result was seen in group II. But, in group III out of 20 chicken 16 chickens were died. Both group I and group II had same protection efficacy with survival rate of 87.5% (Table 5).

Table 5: Protective efficacy evaluation of inactivated fowl cholera vaccine

Group	Chickens(#)	No of chicken survived (%)	No of chicken died (%)	Protective efficacy
I	20	18(90%)	2(10%)	87.5%
II	20	18(90%)	2(10%)	87.5%
III	20	4(20%)	16(80%)	-

The numbers of chickens showing clinical signs of fowl cholera were presented in table 6. However, there is significant difference in the number of chickens showing the clinical signs between vaccinated (Group I and II) and control (Group III). In group III, 100% of the chickens showed visible clinical signs like depression, ruffled feathers, loss of appetites, greenish diarrhea, conjunctivitis, and cloudiness of the eye with unilateral or bilateral blindness, labor breathing, and lameness with swollen joint in some cases with death starting from the third day of challenge whereas only 15% in group I and 20% in group II showed clinical signs of fowl cholera.

Table 6: Number of chicken showed clinical sign of fowl cholera, after post challenge dose.

Group and No of chickens	Dose	Greenish diarrhea	Lameness	Conjunctivitis	Ruffled feathers	Labor breathing	Total No of chickens showing any of the signs
I=20	1ml	2	3	2	2	2	3
II=20	0.5ml	2	3	1	4	4	4
III= 20	contr ol	20	20	8	20	20	20



Figure 3: Clinical sign of fowl cholera during challenge experiment in the control group

For confirmation of the cause of death all chickens that died during the course of the experiment were sampled for microbiological examination and *P. multocida* was re-isolated from internal organ (liver, spleen and heart) and subsequently identified both phenotypic and PCR technique. The re-isolated *P. multocida* from chickens were gram negative short rod-shaped and occur singly or in pairs. In blood and nutrient agar media, the organism produced characteristically whitish, discrete, opaque, circular, convex culture and translucent in appearance and the culture of spleen and liver of dead chickens onto typtose agar revealed typical colonies of *P. multocida*. Some biochemical tests conducted like oxidase, catalase and indole were positive and do not show hemolysis. Once the colony morphology, staining characteristics and, some biochemical tests were positive, confirmatory identification was done by PCR seen in (Figure 5).

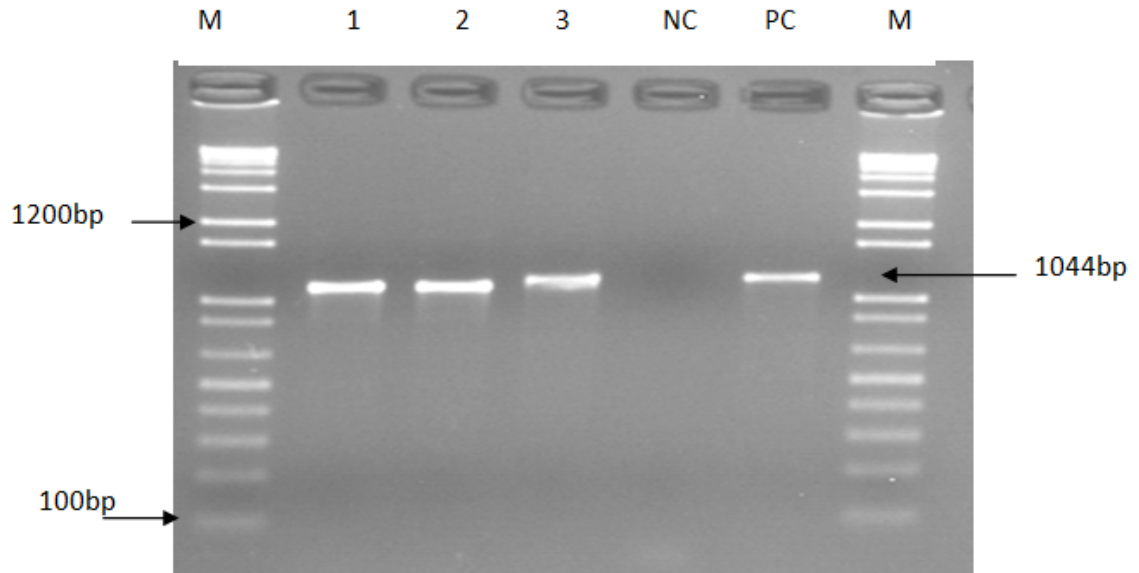


Figure 4: Result of gel electrophoresis of *P. multocida* type A re-isolated M, molecular marker (started 100bp up to 1kb plus, invitrogen), (Lane 2) vaccine seed used positive for capsular type A 2 (Lane 3) liver positive for capsular type A; 3- (Lane4) spleen positive for capsular type A; 4 (Lane 5) negative control without template 5 (Lane 6) positive control.

4. DISCUSSION

In the present study, a higher antibody titer was observed in both vaccinated groups (Group I-1ml vaccinated and group II-0.5ml vaccinated) at post vaccination and the differences in antibody titer was statistically significant as compared to day 0 antibody titer. Similar result was reported by Islam *et al.* (2004) who stated that IHA antibody titer in ducks immunized by fowl cholera vaccine was found significantly increased post vaccination. In Group III (control), the value of the IHA antibody titer remained constant throughout the experimental program proving that the increase in antibody titer was due to specific antibody response to the vaccine (Table 3). In group I and II the antibody titer showed a significantly increase ($p < 0.001$) from day zero to day twenty one. Furthermore, there was a significant rise in antibody titer after booster dose up to 35 day with ($P < 0.001$) this result closely related with the finding of Modak *et al.*, (2012) who also found a statistically significant increase in antibody titer in chicken immunized by fowl cholera vaccine after secondary vaccination.

When compared IHA antibody titer between group I and group II, almost similar antibody titer were seen at day 14 and day 21. However, in group II antibody titer at day 28 slightly higher than group I. Group I antibody titer at day 35 increase to some extent than group II but the differences were not statistically significant (Table 3). According to Choudhury *et al.* (1990) immunized chickens using formalin killed alum precipitated fowl cholera vaccine prepared with *P. multocida* and administered twice at two weeks interval and observed immunoglobulin levels increased in double vaccinated groups of birds and similarly comparable result were observed in present study. Our finding is also in concordance with Wu *et al.*, (1986) who indicated that two doses were required with an interval of two to four weeks and with inactivated vaccines full immunity could not be produced until approximately two weeks after the second dose of a primary vaccination. Likewise, in present study, after booster dose a high immune response was observed indicating the need for booster dose in vaccination programs using inactivated fowl cholera vaccine.

In present study, the efficacy of the vaccine was further evaluated by challenging the vaccinated and control chickens with the virulent strain of *P. multocida* isolated from field outbreak. The immune responses of chickens vaccinated with 1ml dose (Group I) and 0.5ml (Group II) were similar in terms of protection rate against *P. multocida* in which 87.5% of protective efficacy was obtained in both vaccine doses. The present result agreed with Islam *et al.* (2004) in which immunized ducks with Bangladesh Agricultural University fowl cholera vaccine showed a 90% survival rate after challenge infection within three weeks after vaccination. After 7 days of challenge infection, all chicken in control group (Group III) showed symptoms of fowl cholera and 16 chickens died with 80% mortality rate starting from day three. This higher mortality recorded in the control group of the present study was comparable to previous study conducted in Ethiopia where mortality of 85% was recorded in control groups (Bitew *et al.*, 2009). Similarly, the clinical sign of fowl cholera in control group observed during challenge experiment agreed with previous studies reported by (Islam *et al.*, 2017; Akhtar *et al.*, 2016). On the other hand, in vaccinated groups only three in Group I and four chicken in Group II, showed clinical sign of fowl cholera (Table 6) and the mortality rate was only 10% in group I and II (2 from group I and the other 2 from group II).

Protective efficacy in vaccinated groups result was similar and the immune responses of chickens vaccinated with 1ml/chicken and 0.5 ml/chicken acceptable in terms of protection rate against *P. multocida*. This result also similar with Islam *et al.* (2017) in which the immune responses of pigeons vaccinated with 0.4 ml/bird, 0.6 ml/bird, 0.8 ml/bird and 1 ml/bird were satisfactory in terms of protection rate against *P. multocida*. In addition to this, vaccination with 0.5 ml dose has been observed in providing significant protection in terms of inducing immune response as reported by Parvin *et al.*, (2011) and Modak *et al.*,(2012) where inoculation of 0.5ml/birds fowl cholera vaccine induced significant serum antibody determined by indirect haemagglutination test. With respect to cost and vaccine usage, vaccination at lower the dose rate of 0.5ml was preferred for the field study.

In the field vaccine evaluation study, similar to the on-station result the antibody titer increased significantly at day 21 after primary vaccination as compared to pre-vaccination titer. Moreover, IHA antibody titer increased significantly ($P < 0.001$) after booster dose was given (Table 4). Like on-station study, after booster dose, the high immune response indicating the need for booster dose in vaccination programs using inactivated fowl cholera vaccine at field condition. This result was comparable with Bag *et al.*, (2015) who reported that vaccination of fowl cholera vaccine with 0.5ml doses produced higher level of antibody when booster dose was given after primary vaccination at farm level.

5. CONCLUSION AND RECOMMENDATIONS

In Ethiopia, fowl cholera causes a significant economic loss to the growing poultry sector, hence, developing and producing protective and efficient vaccine from locally isolated *P. multocida* is vital to control this disease. The present study based on on-station and field evaluation results, confirmed that inactivated fowl cholera vaccine produced at National Veterinary Institute from locally isolated *P. multocida* was safe, immunogenic with high level of protection efficacy (87.5%) against fowl cholera in chicken. In addition, the result also concluded that the vaccine produces better protection after booster vaccination and protection efficacy of 1ml and 0.5ml dose of vaccine had similar performance.

Based on the above conclusion the following recommendations are forwarded

- ✓ The stability, duration of protection, shelf life of the inactivated fowl cholera vaccine should be studied.
- ✓ Field evaluation of fowl cholera vaccine should be done under different management and breed of chickens
- ✓ Further field survey and outbreak investigation is essential to collect *P. multocida* isolates from different parts of Ethiopia for screening widely distributed genotype or strain and possible consideration as vaccinal candidates
- ✓ The National Veterinary Institute, a veterinary vaccine producer in Ethiopia, should consider the results of this research for potential use and production of inactivated fowl cholera vaccine to control fowl cholera in the country and save foreign currency used to import other fowl cholera vaccines from abroad.
- ✓ Use of 0.5ml vaccine dose with booster vaccination at day 21 after primary vaccination should be recommended.

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7. ANNEXES

Annex 1: Media used for bacteriological examination

1. Tryptic Soy Agar (Difco,spark,USA) composition and preparation.

Composition of Tryptic Soy Agar	per liter of deionized water
Pancreatic Digest of Casein	15.0gm
Peptic Digest of Soybean Meal	5.0gm
Sodium Chloride	5.0gm
Agar	15.0gm

Final pH = 7.3 +/- 0.2 at 25°C

2. Tryptic soy broth (Oxoid Ltd.,Hampshire, England) composition and preparation.

Composition of Tryptic Soy Agar	per liter of deionized water
Pancreatic Digest of Casein	17.0gm
Peptic Digest of Soybean Meal	3.0gm
Sodium Chloride	5.0gm
Dipotassium Phosphate	2.5gm
Dextrose	2.5gm

Final pH = 7.3 +/- 0.2 at 25°C.

3. MacConkey Agar (Oxoid, Hampshire, England) composition and preparation.

Composition of MacConkey Agar	per liter of deionized water
Peptone (Pancreatic digest of gelatin)	17 gm
Proteose peptone (meat and casein)	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Crystal Violet	0.001 g
Agar	13.5 gm
Neutral red	0.03 gm
Sodium chloride	5 gm
Final pH = 7.1 +/- 0.2 at 25°C.	

4. Vian-de foie(VF)media

Preparation and composition of Vf media

Chemical composition	unit	Quantity
Glucose	Gram	5
L-cystine	Gram	0.5
Meat extracts Broth	Liter	1
Yeats extract	Gram	3
Minced meat	Gram	4
Liver cooked	Gram	1
Paraffin oil	milliliter	1

5. Blood Agar media composition and preparation.

Composition of Blood Agar media	per liter of deionized water
Beef extract	10.0 gm
Agar	15.0 gm
Peptone	10.0 gm
Sodium Chloride (NaCl)	5.0 gm
Sheep blood, defibrinated	50.0 ml

Final pH = 7.3 +/- 0.2 at 25°C.

Annex 2: Fowl cholera Inoculum and production media

No	Chemical type	Unit	Quantity in litter	Remak
1	Peptone	Gram	10	
2	Casitone hydrolysate	Gram	5	
3	Sodium chloride	Gram	5	
4	Di sodium hydrogen sulphate (Na ₂ HPO ₄)	Gram	7	
5	Potassium Di hydrogen sulphate (KH ₂ PO ₄)	Gram	2.5	
6	Magnesium sulphate(MgSo ₄ 7H ₂ O)	Gram	1	
7	Yeast extract	Gram	2.5	
8	D (+) glucose	Gram	5	
9	Horse serum	ml	10	10%
10	Distilled water	ml	990	

Source: OIE, 2000

Annex 3: Procedures of some biochemical test

1. Catalase test (Quinn et al.,2002)

Principle: The breakdown of hydrogen peroxide into oxygen and water is mediated by the enzyme catalase.

Procedure: A loop full of the bacterial growth is taken from the top of the colonies avoiding the blood agar medium. The bacterial cells are placed on a clean microscope slide and a drop of 3% H₂O₂ is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.

2. Oxidase test (Quinn et al.,2002)

Principle: The cytochrome oxidase enzyme is able to oxidize the substrate tetramethylpphenylenediamine dihydrochloride, forming a colored end product, indophenol.

Wet Filter Paper Method:

- a. A strip of filter paper was soaked with a little freshly made 1% solution of the reagent (tetra methylene p- phenylene dihydrochloride).
 - b. A single colony was rubbed on it with a glass loop. Deep blue color was observed in positive reaction within 5-10 seconds, a “delayed positive” reaction by colouration in 10-60 seconds, and a negative reaction by absence of colouration or by colouration later than 60 seconds. *Pseudomonas aeruginosa* can be used as a positive control organism.
3. Indole test

Principle: Indole positive bacteria possess an enzyme tryptophanase, which converts tryptophan to indole.

- a. Took sterilized test tubes containing 2 ml of peptone water.
- b. Tubes were inoculated with suspected colony growth from 18 to 24 hrs culture
- c. Tubes were incubated at 37°C for 24-28 hours.
- d. 0.5 ml of Kovac’s reagent was added to the broth culture.
- e. Presence or absence of ring formation was assessed with naked eye

Interpretation: The formation of dark red ring indicates positive reaction while in negative reaction a yellow ring is formed.

Annex 4: Preparation of challenge strains

P. multocida strains were used as challenge strains in the test of the vaccines. Freeze-dried stocks were reconstituted with 2 ml tryptose broth (TB) and suspensions were streaked on tryptose agar (TA) plates incubated for 24 h at 37°C. From the culture on TA, a typical colony was inoculated to 50 ml TB and incubated for 7 h at 37°C. These cultures were then adjusted spectrophotometrically at 450nm to 0.475 OD value, and serially diluted to 10^{-1} , 10^{-2} and 10^{-3} in TB. The dilutions were evaluated for pathogenicity by challenging chickens in a pilot trial after which the optimum challenge dose was

determined for use in the challenge experiment. The titre of the culture used for pilot trial was determined by titration (Quinn *et al.*, 2002)

Annex 5: DNA extraction:

DNA was obtained from the isolates using boiling method mentioned by QueipoOrtun *et al.* (2008) with slight modification. Briefly, the organisms were cultured onto typtose agar at 37oC for overnight aerobically. Then, a loopful of bacteria was picked up by swiping and mixed in 200 µL of deionized water. The mixture was then heated in boiling water for 10 min followed by dipping into ice for 10 min and centrifugation was done at 13,000 rpm for 10 min. The supernatant was collected and stored at -20°C until used.

Annex 6: Master Mix Preparation

Ser.no	Type of reagent	For one reaction	Total reaction	6	Remark
1	RNase free water	3 µl	18 µl		
2	Primer-FC-Cap A-Fow-5pm/ µl -5'-TGCCAAAATCGCAGTCAG-3'	2µl	12µl		
3	Primer-FC-Cap A-Rev-5pm/ µl -5'-TTGCCATCATTGTCAGTG-3'	2µl	12µl		
4	IQsupermix	10µl	60µl		
5	Add template(DNA)	3µl			
	Total volume	20µl			

Annex 7: Agarose Gel Preparation

Prepare 1.5% Agarose gel

- ✓ Add 4 μ Gel red with Loading dye
- ✓ 10 μ l PCR product and 10 μ l markers (Ladder)
- ✓ Run Electrophoresis for 1 hour at 120V
- ✓ Read the result by using UV -light

It is around 1044bp positive result for sero-type A

Annex 8: List of pictures captured during laboratory works

