Development of In-House Indirect ELISA Kit Method for the Serological Detection of Antibodies against Fowl Cholera in Chickens

Amde Zeleke

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Masters in Biotechnology

Office of Graduate Studies

Adama Science and Technology University

October, 2024

Adama, Ethiopia

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DECLARATION

I hereby declare that this Master Thesis entitled "**Development of In-House Indirect ELISA Kit Method for the Serological Detection of Antibodies against Fowl Cholera in Chickens**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citations.

Amde Zeleke _____ _____

Name of student Signature Date

RECOMMENDATION

we, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "**Development of In-House Indirect ELISA Kit Method for the Serological Detection of Antibodies against Fowl Cholera in Chickens**" prepared under our guidance by **Amde Zeleke** submitted in partial fulfillment of the requirements for the degree of Master's of Science in Biotechnology. Therefore, we recommend the submission of revised version of the thesis to the department following the applicable procedures.

Professor Hunduma Dinka _____ _____

Major advisor Signature Date

Takele Tesgera (PhD) _____ _____

Co-advisor Signature Date

APPROVAL SHEET

We, the advisors of the thesis entitled **"Development of In-House Indirect ELISA Kit Method for the Serological Detection of Antibodies against Fowl Cholera in Chickens"** by Amde Zeleke, hereby certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Professor Hunduma Dinka _____ _____

Major advisor Signature Date

Takele Tesgera (PhD) _____ _____

Co-advisor Signature Date

We, the undersigned, members of the Board of Examiners of the thesis by Amde Zeleke have read and evaluated the thesis entitled "**Development of In-House Indirect ELISA Kit Method for the Serological Detection of Antibodies against Fowl Cholera in Chickens**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

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LIST OF ABBREVATIONS AND ACRONYMS

AGID	Agar Gel Immuno Diffusion
BCA	Bicinconic Acid
CV	Coefficient Variation
ELISA	Enzyme Linked Immune Sorbent Assay
HRP	Horse Reddish Peroxidase
IHA	Indirect Hemagglutination Assay
LPS	Lipopolysaccharide
NVI	National Veterinary Institute
OD	Optical Density
OIE	Office of International Des Epizooties
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Tween
PCR	Polymerase Chain Reaction
TA	Tryptose Agar
TB	Tryptose Broth
UV	Ultra Violet
WOAH	World Organization of Animal Health

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SUMMARY

Fowl cholera is an infectious disease of birds caused by gram negative *Pasteurella multocida.* It is endemic to Ethiopia and has been causing major economic losses due to mortality and loss of production. Owing to the lack of appropriate diagnostic methods, the exact circulation of Fowl cholera in Ethiopia has been not well studied and documented. Thus, the present study is aimed to develop an in-house indirect Enzyme Linked Immuno-Sorbent Assay (ELISA) kit method that can detect antibodies produced against Fowl cholera. For this purpose a freeze-dried Fowl cholera seed obtained from National Veterinary Institute's (NVI) stock was allowed to grow on tryptose media. The purity and identity of the *Pasteurella multocida* was confirmed after being subjected to Gram stain and Polymerase Chain Reaction respectively. The bacterial cells were lysed by high speed centrifugation through washing by Phosphate buffer saline and the antigen was purified and the protein content was estimated using the Bicinconic Acid assay method. In this study all data were analyzed by Microsoft excel sheet of window 10. The working antigen concentration, serum dilution and secondary antibody dilutions were optimized at 1.5mg/ml, 1:600 and 1:2000, respectively. The incubation time and temperature were optimized at 25^0C (room temperature) for 30 minutes. On the other hand, the cut-off value of the in-house ELISA test was determined to be 0.28. The test sensitivity and diagnostic specificity of this assay was found to be 89.9% and 95%, respectively. The intra and inter assay comparisons within wells and between the plate falls within the acceptable range. Therefore, the newly developed ELISA kit method is reliable, sensitive, specific and can be used for the detection of antibodies against Fowl cholera. It is recommended that, further work should be carried out to confirm the assay's sensitivity, specificity, and repeatability using large number of field samples.

Keywords: *Antibody, Antigen, Chicken, ELISA kit, Fowl cholera, Pasteurella multocida.*

1. INTRODUCTION

1.1. Background

Fowl cholera is a commonly occurring infectious disease caused by the gram negative, non-motile, aerobic and coccobacilli bacterium *Pasteurella multocida* which belongs to *Pasteurellaceae* family and affects all types of birds globally (Huberman, 2016). Bacterial species that belongs to this family are classified into five serogroups (A, B, D, E, and F) and 16 somatic serovars. *P. multocida*, which typically consists of strains of capsular serogroup A and serovars 1, 3, and 4, causes Fowl cholera disease in chickens (Luo *et al*., 2019). *P. multocida* subspecies *septica* and *gallicida* may also cause Fowl cholera like disease to some extent (Christensen and Bisgaard, 2008).

In 1881, *P.multocida* was first identified to be the causative agent of Fowl cholera disease by Louis Pasteur, then bacterium has been known as the causative agent of many other economically important diseases in a wide range of hosts (Harper and Adler, 2006). The outbreak of Fowl cholera often occurs as an acute fatal septicaemia, commonly in adult birds (WOAH, 2021). Mainly it colonizes the respiratory tract of birds and causes stress under respiratory conditions. Fever, anorexia, mucus discharge, ruffled feather and watery diarrhea are the common clinical signs. Chronically, it causes lameness, swollen wattles and torticollis, but it can also be asymptomatic (Niemi, 2021).

The disease is prevalent in most regions of Ethiopia and results in major economic losses due to decreased production and its high mortality (Dessalegn *et al*., 2021). In natural outbreaks, Fowl cholera is mostly diagnosed by conventional methodologies including the isolation and identification by serotyping and biochemical characterization, which demonstrates the presence of variable serogroups/types in different geographical regions. However, conventional diagnostic methods has been observed to be not sensitive enough to identify and differentiate agents involved in natural infection (Harper and Adler, 2006). Vaccination and treatment by antibiotic have been essential method for successful control of Fowl cholera globally (Baksi *et al*., 2018).

Accurate and timely diagnoses are regarded as effective tools in controlling infectious disease like Fowl cholera. Different serological tests, such as agglutination, Agar Gel Immuno-

diffusion Assay **(**AGID) and passive hem agglutination have been employed to detect antibody against Fowl cholera in serum from chicken hosts. However, none of them were highly sensitive (WOAH., 2021).

Enzyme-linked immunosorbent assay (ELISA) tests for antibody titer determination have been employed with variable degrees of success in attempts to monitor sero-conversion in vaccinated poultry, but not for diagnosis (Poolperm *et al*., 2017). Among the serological tests, ELISA is considered as a rapid, highly sensitive, specific and effective diagnostic tool for antibody detection (Shah & Maghsoudlou, 2016). Several different antigens of *P. multocida* such as, whole cell antigen, sonicated antigen, potassium thiocyanate extract, lipopolysaccharide, sodium salicylate extract and capsular or heat extract antigen have been generated for coating ELISA plates (Afzal *et al*., 1992; Tankaew *et al*., 2018).

1.2. Statement of the problem

Fowl cholera is the major infectious disease that affects poultry industry in developing countries like Ethiopia (Wubet *et al*., 2019). Considering the significant impact of the disease, specific control strategies including treatment and vaccination depends on accurate diagnosis. Thus, the absence of early and reliable diagnostic techniques hinders effective treatment, control and prevention measures.

ELISA is valuable due to its rapidity, sensitivity and specificity. However, the commercially available ELISA kits are very expensive and take time for delivery making it difficult for professionals to detect early the antibodies of the agents to control and prevent the prevalence of the disease in poultry flocks (Bitew *et al.*, 2009). Thus, looking for a locally (In-house) produced diagnostic method that can detect antibodies against Fowl cholera is paramount task for researchers in developing country.

1.3. Significance of the study

Timely and accurate diagnosis of Fowl cholera plays a crucial role in managing the disease to safe guard the health and productivity of poultry flocks. Blood tests such as Enzyme-linked immunosorbent and agglutination assays are used to detect the antibodies of causative agents, *P. multocida*. Thus, developing an in-house indirect ELISA kit for the accurate and sensitive

detection of Fowl cholera antibodies in chickens helps to address a reliable tool to enhance early detection and control of disease outbreak in poultry flocks.

Accordingly, the current study helps to minimize the expenditure that will be spent for the purchase of commercial kit by poultry farms such as reduces shipping and import costs, making the kit more affordable for researchers, poultry farms, and institutes like NVI and others. It will be also used for teaching purpose in different research and higher learning institutes and support local economy by contributing the growth of biotechnology sector within the country. Additionally, the newly developed kit allows the assessment of the sero-conversion against Fowl cholera and optimization of the assay based on the available resources. In general, locally developed ELISA kit can enhance the efficiency, accessibility and relevance of research activities within the specific context of local researchers and institutes**.**

1.4. Objective

The objective of this study was to develop an in-house indirect ELISA kit method that can detect antibodies against Fowl Cholera in chickens, in Ethiopia.

2. LITERATURE REVIEW

2.1. Description of Fowl cholera

Fowl cholera is a contagious bacterial disease of birds caused by *Pasteurella multocida*. Acutely, it causes an elevated mortality rate. Chronically, it may cause lameness, swollen wattles (in chickens), pneumonia (in turkeys), and/or torticollis; however, birds can also be subclinically affected carriers. Both live, attenuated vaccines and adjuvanted bacterins are available to aid in prevention, and the organism is susceptible to some antimicrobials (https://www.msdvetmanual.com/poultry/fowl-cholera/fowl-cholera). Avian pasteurellosis and Avian haemorrhagic septicaemia are two common synonyms for Fowl cholera (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. Etiology

P.multocida, 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 Fowl cholera 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.multocida's* antigenic properties. Fowl cholera-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 Fowl cholera cause 80% mortality, but type D strains of Fowl cholera cause 20% mortality (Modak *et al*., 2012).

2.3. Features 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 Fowl cholera 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, 2019).

2.4. Epidemiology

2.4.1. 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).

2.4.2. Host range

Fowl cholera 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, Fowl cholera is not regarded to have zoonotic potential (OIE, 2018).

2.4.3. 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 Fowl cholera (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.

2.4.4. Transmission and risk factors

The presence of feather shafts in the ventricular lumen of most bird family carcasses diagnosed with Fowl cholera shows that scavenging or cannibalism of Fowl cholera-dead birds is a primary mechanism of transmission (Wille *et al*., 2016). The occurrence of Fowl cholera can be linked to monthly mean temperature, relative humidity, and rainfall with a 2-3 month lag. Climate variability plays an essential role in Fowl cholera transmission and the model of strong predictive capacity for the occurrence of Fowl cholera (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 (Table 1) (Wobeser, 1997). The infection does not spread vertically through the egg (Glisson, 2008).

Table 1: The potential transmission of Fowl cholera to wild birds

Route of transmission and field situation	Comments
Bird-to-bird contact	The transmission of Fowl cholera 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 Fowl cholera epidemic and then redirected for other purposes.

2.5. 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 pseudo-tuberculosis, 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 Fowl cholera (OIE, 2018).

2.5.1. Clinical signs

Depending on how far the disease has progressed, the clinical symptoms of Fowl cholera 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 & Harshfield, 1953).

Signs and symptoms of chronic Fowl cholera are usually associated with localized infections of the sternal bursa, wattles, joints, tendon sheaths, and footpads, which are often enlarged due to accumulated fibrino suppurative 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).

2.5.2. 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 hemorrhages. Some of the lesions caused by Fowl cholera 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).

2.5.3. 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, minositol, 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. The tests and results listed in table 2 may usually be used to distinguish *P. multocida* from other avian *Pasteurella spp*. and *Riemerella anatipestifer* (OIE, 2018).

Table 2: Test used to differentiate *P. multocida* from other Avian Pasteurella species and *Riemerella anatipestifer.*

Test tools	*Pasteurella*		*Riemerellaanatipestifer*
	multocida	*gallinarum*	
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.

2.5.4. Diagnosis of Pasteurella multocida by serological and molecular methods

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; 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 Immuno-diffusion *(AGID)* test is commonly used to determine somatic serotypes. 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 was 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; Sultana *et al*., 2013).

The ELISA assay was used for investigation of antibody to Fowl cholera in avian species, and the results were compared to standard IHA test and micro titer agglutination (MA) test. The sensitivities of those ELISA tests were higher than both IHA and MA tests (Marshall *et al*., 1981, Solano *et al*., 1983). This suggested that the ELISA test is a more effective serological test for Fowl cholera antibody detection in avian species.

PCR techniques

Phenotypic differentiation tools have been mostly replaced by genotypic methods (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).

A specific DNA sequence of the pathogen genome is amplified and the generated PCR-product is detected by an oligonucleotide-probe labelled with a fluorescent dye. This technology allows for a sequence-specific detection of PCR amplicon. The *P. multocida* qPCR Kit is intended to detect *P. multocida* genomes in vitro. The kit is designed to be as broadly specific to *P. multocida* as possible while maintaining its broadest detection profile. Based on a thorough bioinformatic analysis using all reference data from the NCBI database, the primers and probe sequences exhibit very high (>95%) homology with a wide range of *P. multocida* genomes (Loy *et al*., 2018).

Restriction Endonuclease Analysis

The restriction endonuclease enzymes used in restriction endonuclease analysis (REA) are a typical component of the "house maintenance" system of bacterial cells. When the enzyme's particular recognition sequence is met, the enzymes can cleave double-stranded DNA. REA of avian *P. multocida* has typically been carried out with six- or four-base cutter enzymes. A list of the enzymes that have been used to date is *Bgl*II, *Eco*RI, *Hha*I, *Hpa*II,*Pst*I, *Sma*I, *Sma*I/*Sal*I and *Xho*I having different recognition sequence site. Thus, several studies have been reported on the use of REA of avian *P. multocida* using these enzymes (Snipes *et al*., 1990, Christiansen *et al*., 1992a).

Ribotyping

Ribotyping is a typing technique based on REA. On the other hand, just specific DNA fragments are seen rather than viewing every DNA fragment produced on a standard REA gel. In order to accomplish this, the usual REA analysis's gel electrophoresis must be finished, and the DNA fragments must then be transferred from the gel onto a membrane surface (usually nylon). Afterwards, a nucleic acid probe that only binds to DNA fragments containing segments similar to the probe is exposed to this membrane. The term "ribotyping" refers to the visualization technique wherein the probe is derived from either the ribosomal RNA (rRNA) or its coding genes. The banding pattern produced by ribotyping is significantly simpler than that produced by REA.

The various enzyme and probe combinations that have been used in ribotyping avian isolates of *P. multocid*a are *Eco*RI (G↓AATTC), *Hha*I (GCG↓C), *HinD*III (A↓AGCTT), *Hpa*II (C↓CGG), *Hpa*II (C↓CGG), *Hpa*II (C↓CGG) and *Pst*I (CTGCA↓G) (Blackall, 2000).

2.6. Poultry industry in Ethiopia

The world's largest livestock group, estimated at 23.39 billion, is poultry, which mostly consists of chickens, ducks, and turkeys. Poultry has continued to play a significant role in improving food security and livelihood, accounting for between 28 and 30 percent of all animal protein consumed worldwide (ILRI, 2014). In Ethiopia, according to report of Central statistical agency, there are about 57 million total poultry population, including cocks, cockerels, pullets, laying hens, non-laying hens, and chicks. Out of the total chickens mentioned, indigenous poultry breeds account for the majority at 78.85%, while hybrid and exotic breeds make up 12.02% and 9.11% respectively (CSA, 2021).

According to scholars, Ethiopian poultry production can be divided into small, medium, and large categories, with conventional and underdeveloped value chains. Studies have indicated that, particularly in smallholder production systems, the poultry value chain distribution technique has proven to be more effective than the input-output approach (Hailemariam and Zemedu, 2018).

Despite significant potential of the sector, the per capita consumption of poultry products in Ethiopia is low compared to other East African countries, with an annual average of just 57 eggs and 2.85 kg of chicken meat per person. To support and expand the sector, the Ethiopian government has laid out a plan to increase egg production from the current 2.8 billion to 5.5 billion by 2029, and raise poultry meat's share in the national diet from 9% to 30% by 2030. This plan also includes encouraging poultry product exports, as Ethiopia exported around 35 tons of live chickens to Somalia in 2020. However, challenges such as disease management, inadequate poultry feed, and lack of access to quality production equipment limit rapid expansion (https://ethiopoultryexpo.com/wp-content/uploads/2024/08/ETHIOPEX-2024Brocuhre.pdf, accessed on October 31, 2024).

However, the poultry sector in Ethiopia continues to face challenges from infectious diseases like Newcastle disease, Infectious bursal disease, coccidiosis, and fowl cholera, which collectively impact productivity. Addressing these with improved vaccine strategies, could substantially reduce losses and improve poultry health across Ethiopia's diverse farming settings(Asfaw *et al*., 2019).

2.7. Status of Fowl cholera in Ethiopia

Despite its rapid growth, Ethiopia's poultry industry has several challenges, particularly infectious diseases, which have seriously impact on the development of the sector. Fowl cholera (FC), caused by *Pasteurella multocida*, remains a significant poultry disease in Ethiopia, affecting poultry production across various regions. The disease is endemic in Ethiopia and has been reported to cause significant economic losses due to high mortality, especially in large flocks, and reduced production in affected poultry operations (Gebre-gziabher, 2007).

In Ethiopia, to control the disease, the widely used fowl cholera vaccine is the formalin-inactivated fowl cholera (FI-FC) vaccine, produced locally by the National Veterinary Institute (NVI) in Bishoftu. Regardless of its availability, challenges with this vaccine remain due to its limited ability to induce long-lasting immunity. Despite the use of formalin-inactivated vaccines, the disease persists, largely due to limitations in the vaccine's efficacy, which is relatively short-lived and less effective in inducing mucosal immunity. This has led to increased interest in developing more effective alternatives, including gamma-irradiated vaccines that stimulate a stronger and longer-lasting immune response, critical for preventing avian infections. Trials using gamma-irradiated FC vaccines have shown promise, with intranasal or intraocular administrations achieving higher protection levels compared to traditional methods (Dessalegn *et al*., 2021).

2.8. 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 Fowl cholera. 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 Fowl cholera is already prevalent and causing problems on the farm (Jonas *et al*., 2001).

2.8.1. 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). Fowl cholera 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 aluminum 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 Fowl cholera, 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. MATERIALS AND METHODS

3.1. Laboratory Facilities

The study was conducted from November 2023-August 2024 at Serology and bacteriology Laboratory of National Veterinary Institute (NVI) which is found at Bishoftu, Ethiopia. The serology laboratory is certified with ISO/IEC 17025 and able to generate valid test results. Culture of the organism and purity test were done at bacteriology laboratory and serological assays were carried out at serology laboratory. The laboratory is equipped with the requirements of biosafety level 2 and biological safety cabinet is used as primary barrier for safety. The chemicals and bacterial splashes may be formed during standard work procedures like mixing, pippetting and centrifuging, so these activities were performed under biological safety cabinet. However, most of the serological assays were conducted on open field.

3.2. Culturing of the bacteria and antigen preparation

One vial freeze dried local isolate vaccinal strain of *P.multocida* type A, obtained from NVI, was reconstituted in sterile Tryptose broth (TB) media (*HIMEDIA, India*) and streaked on tryptose agar (TA) (*SIGMA-ALDRICH, German*). The streaked plate was incubated overnight at 37^0c to achieve bacterial growth from freeze dried culture (NVI Fowl cholera vaccine production protocol). Once growth was confirmed through visualization of the colonies, purity was also checked by the gram stain for the presence of pure gram negative *coccobaccilli* organisms.

After the purity of *pasteurella multocida* was confirmed, the second passage was carried out by inoculating fresh colony from TA to sterile TB media by incubating at 37^0c for 7 hrs. Then the growth and the purity of bacteria were checked by its turbidity and gram stain technique, respectively. Consequently, the seed was inoculated into 2 bottles of 1 liter sterile inoculums containing 5% of horse serum and glucose and incubated at 37^0c for 18 hrs. Again, following the confirmation of the growth, purity was checked by gram staining and PH value (NVI, protocol).

3.2.1. DNA extraction

DNA extraction was performed using a Qiagen extraction kit according to the manufacturer's instruction (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6425773/). Briefly, bacterial susp ension of 200µl, 20 µl of proteinase K and 200µl of AL buffer were added and homogenized by vortex for 15 seconds and then incubated at 60 ˚C for 10 minutes. A total of 200 µl 96-100% ethanol was added and homogenized. Then, the suspension was transferred to mini spin column, and centrifuged at 8,000 rpm for 1 minute, the flow through was removed and 500 µl washing buffer (AW1) was added and centrifuged at 8,000 rpm for 1 minute. The flow through was discarded and 500 µl AW2 washing buffer was added and centrifuged at 14000 rpm for 3 minutes. The mini spin column was placed in the 2 ml collection tube and centrifuged again for 1 minute at 14000 rpm to dry the column matrix. The mini spin column was placed in the labeled eppendorf tubes and eluted the DNA by adding 50µl elution buffer by centrifuging at 8000 rpm for 1 minute. The eluted DNA was stored at -20˚C until processing.

3.2.2. PCR assay and gel electrophoresis

Following DNA extraction, *P. multocida* type A causing Fowl cholera was carried out using the PCR technique to amplify a specific fragment of the hyaD-hyaC gene of *P. multocida* using the primer pairs CAPA-FWD (5′- TGC-CAA-AAT-CGC-AGT-CAG -3′) and CAPA-REV (5′- TTG-CCA-TCA-TTG-TCA-GTG -3′) as described by WOAH (2021) with the PCR product approximate size of 1044bp. A total reaction volume of 22µl was used containing 3µL of nuclease free water, 10 µL kits supplied IQ super mix, 2 µL forward primer, 2 µL reverse primer and 5µL template DNA. Thermal profiles are as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; with a final extension at 72°C for 5 minutes. After completing the PCR, 5µL PCR product along with gel red and 6x loading dye was loaded into 2% agarose gel (w/v) for electrophoresis in 1X Tris Acetate EDTA buffer at 120^0C for 1hr using (EC 2060, USA). A 100bp molecular ladder Biotech rabbit GmbH Berlin, Germany, was also loaded and finally read using uvitec Trans illuminator.

3.2.3. Antigen purification and Protein estimation

500ml of the live bacterial culture was taken and centrifuged at 10,000 rpm for 20 minutes. The sediments were washed three times with Phosphate Buffered Saline (PBS) through centrifugation and it was reconstituted by 5ml PBS and was placed at -20^{o}C until protein estimation was conducted. Then, the protein content was estimated by the Bicinconic acid (BCA) assay method using standard (bovine albumin), Copper sulphate ($Cuso_4$), distilled water, and BCA (Lee, 2017).

3.3. Reference serum preparation

3.3.1. Positive serum

Known positive serum was obtained from naturally infected chickens that have been stored in the serum bank of the NVI, serology laboratory and used as a positive control.

3.3.2. Negative serum

Negative serum was collected from non-infected chicken and tested for the absence of antibody against *P. multocida* by using commercial ELISA Kit and used as a negative control.

3.4. Preparation of working solutions

3.4.1. Washing buffer

Washing buffer used in this study was prepared by mixing 500µl of tween 20 in one liter of PBS solution to get the final concentration of 0.05% (0.0005) and homogenized by using a vortex mixer according to method described by (Mohammad and Esen, 1989).

3.4.2. Blocking buffer

5% blocking buffer was prepared by dissolving 5g of skimmed milk (*SIGMA-ALDRICH, German)* in 100 ml of distilled water and the mixture was homogenized by using a vortex mixer (Mohammad and Esen, 1989).

3.4.3. Coating buffer

Coating buffer was prepared by dissolving 3.7gm of sodium bicarbonate ($NaHco_3$) and 0.64gm of sodium carbonate (Na_2co_3) in 1L of distilled water. Its PH was adjusted at 9.4-9.6 (https://www.fortislife.com/products/antibody-resources/sandwich-elisaprotocol/protocol020).

3.5. Optimization of coating antigen and serum dilution

Following protein estimation, the antigen was diluted by coating buffer (bicarbonate buffer) with the concentration of 0.5 mg/ml, 1mg/ml, 1.5 mg/ml and 2mg/ml and was coated into flat bottomed 96 well microplates and incubated at +4 ^oC overnight. Following overnight incubation the microplate wells were washed four times using 300µl wash buffer containing 0.05% phosphate buffered saline tween 20 (PBST). A 200µl of block solution (5% skimmed milk) was added to each well and incubated at 37^oC for 2 hrs to block non-specific bindings. Then the plate was emptied and washed four times as described above and the antigen-coated plate was stored at +4 ^oC until use according to method described by (Minic and Zivkovic, 2020).

The Positive sera obtained from naturally infected chickens that have been stored at serology laboratory of NVI and negative serum was diluted with blocking buffer with the concentration of 1:400, 1:500, 1:600, and 1:1000 and 100µl of the diluted sera was dispensed to each wells vertically and incubated at room temperature for 30 minutes. The excess antibodies were removed and washed with PBST. A 100µl of the horse reddish peroxidase (HRP) conjugated solution prepared in 1:2000 dilution, was added to each well and incubated for 30 minutes at room temperature and followed by emptying and washing of the plate. Then, 100µl of substrate solution (*TMB; Sigma–Aldrich*) was added and incubated for 10 minutes at room temperature (dark place). The reaction was stopped by adding 100µl of sulfuric acid (1N H_2SO_4). The optical density (OD) values were determined using an ELISA reader at a 450 nm filter. The biggest difference between positive and negative serum OD values was selected for antigen concentration and serum dilution, respectively (https://www.bosterbio.com/blog/post/how-to-use-checkerboard-titration-to-optimize-your-elisa-immunoassays).

3.6. Optimization of Secondary antibody (Conjugate)

In this study the secondary antibody was optimized after diluted in the manner of 1:2000 and 1:4000 with blocking buffer. And the indirect ELISA method was applied as above (3.5).

3.7. Optimization of incubation time and temperature

For optimization of incubation time and temperature, positive and negative serum was used and Indirect ELISA technique was applied. The incubation time was compared after being kept for 30 minutes and 1hr for both at room temperature and at 37 ^{o}C according to method described by (Mousavi *et al*., 2016).

3.8. Determination of cut-off value

The cut-off value of the present ELISA technique was determined based on the method reported by Kumar and Rao (1991), using the mean absorbance of negative control plus three times the standard deviation. Thus, to determine the cut-off value, the formula was as:

$$\bar{x} + (3*SD)$$

Where:

$\bar{x}$= the mean of tested samples

SD= Standard deviation

3.9. Evaluation the test sensitivity and diagnostic specificity

The method described by Samad et al. (1994) was used for the determination of the test sensitivity and diagnostic specificity by comparison with the commercial kit. The sensitivity and specificity of the newly developed In-house Indirect ELISA against the commercially available indirect ELISA kit was determined as showed in Table 3.

Table 3: Determination of the test sensitivity and diagnostic specificity by comparison with commercial and in house developed ELISA kit

		Commercially available Indirect ELISA		Total
		Positive	Negative	
In-House	Positive	a	b	a+b
Indirect ELISA	Negative	c	d	c+d
Total		a+c	b+d	a+b+c+d=N

Explanation of the above method:

a =number of samples positive to both In-house and the commercial tests

b = number of samples positive to In-house but negative to the commercial test

c = number of samples negative to In-house but positive to the commercial test

d = number of samples negative to both In-house and the commercial tests

a+b+c+d=Total number of samples (N).

Specificity: The test can detect negative samples, while compared with the commercial test (d/(b+d) x 100)).

Sensitivity: The test can detect positive samples when compared with the commercial (a/(a+c) x 100**)).**

3.10. Validation of the kit

3.10.1. Evaluation of the assay consistency within wells and between the plates (Intra and Inter assay)

The consistency of the assay was checked by the comparison of intra and inter-assay after running the same samples within wells and between plates. The acceptable inter-assay values coefficient of variation (CV) should ideally be less than 15%. Usually, the intra-assay CV value is lower than the inter-assay CV because the variation between runs is higher than on the

same run. The coefficient of variation was formulated using the mean and standard deviation of the OD- values (https://www.abbexa.com/analysing-elisa-data) as follows.

*% Coefficient of variation = (standard deviation/mean)*100*

3.11. Data Management and Analysis

All data that were analyzed by ELISA reader and were entered into MS excel spread sheet program to create data base. Microsoft excel window 10 was used for data analysis and the results were presented in the form of tables and graphs.

3.12. Ethical clearance

In this study laboratory animals were not used. However, the study was carried out according to research ethics and guidelines and Ethical clearance was obtained from National Veterinary Institute (Annex 5).

4. RESULTS

4.1 Antigen preparation and culturing of bacteria

Following the culture of freeze dried local isolate of vaccine strain *P.multocida* type A seed, the growth was demonstrated on TA as spherical and smooth (mucoid) colony and turbidity was observed after inoculating selected colony from agar plate to broth medium and 7 hours incubation (Figure 1 a and b) respectively.

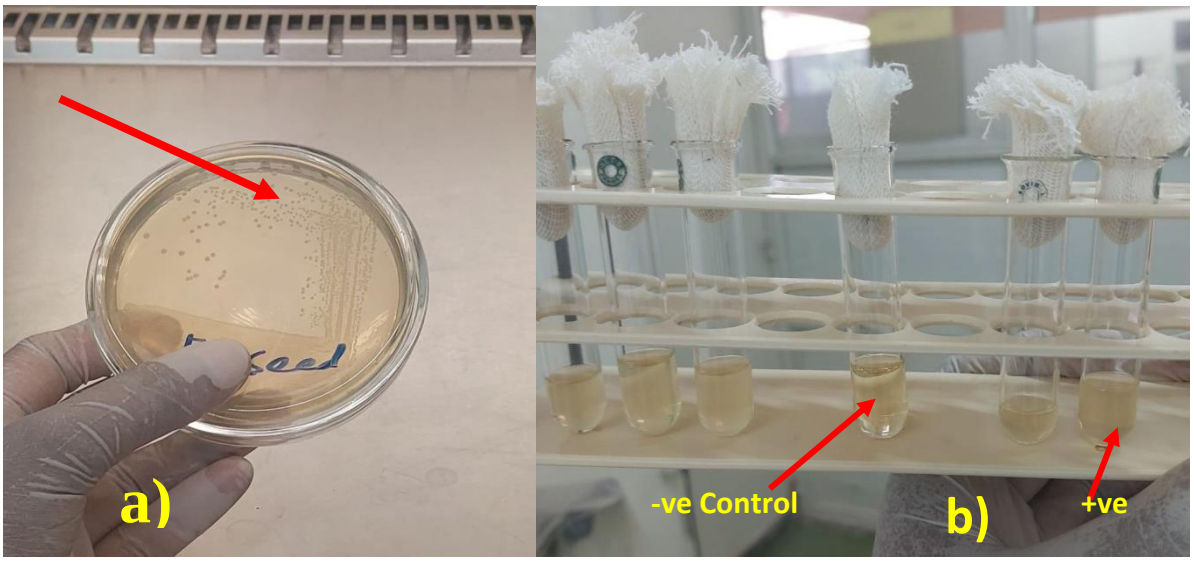


Figure 1: Fresh colony of *Pasteurella multocida* on (a) Tryptose agar media and (b) turbidity of the broth due to bacterial growth

The purity was determined using gram's stain method and was found to have identical morphology of the specific pathogen (Figure 2).

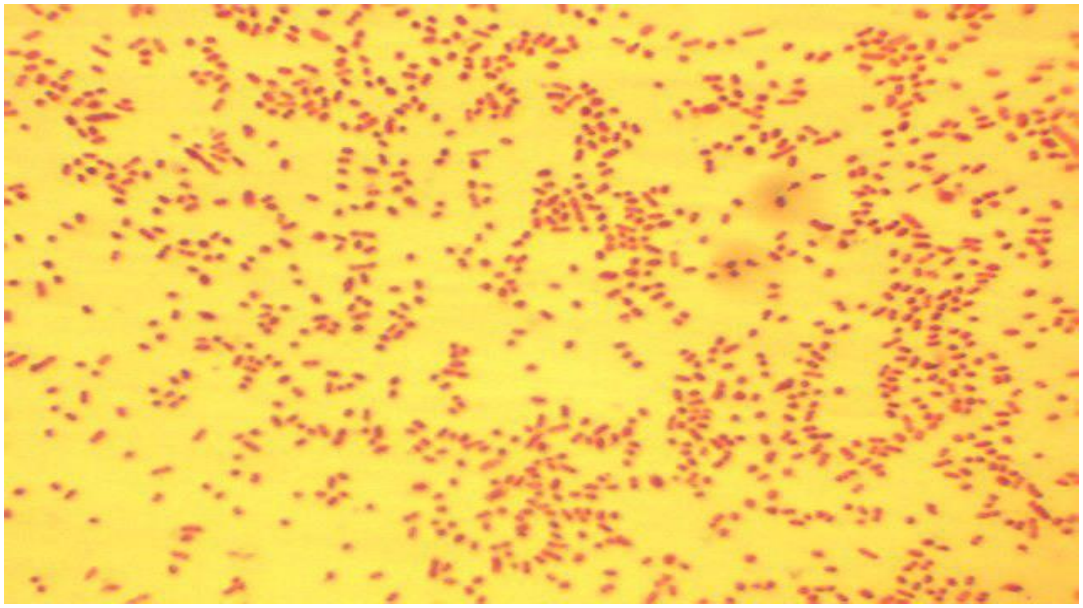


Figure 2: Gram stain of illustrating typical morphology of gram negative *pasteurella multocida* coccobacillary under Motic Moticam 3 plus with 3.0 MP digital microscopy and Motic Images plus software (Hong Kong, China)

4.2. Molecular identification of *Pasteurella multocida*

The pure growth of bacteria was processed for molecular characterization through polymerase chain reaction (PCR). Accordingly, the agarose gel electrophoresis result revealed 1044bp band size, which was considered as the size of antigen of interest (*Pasteurella multocida*) (Figure 3).

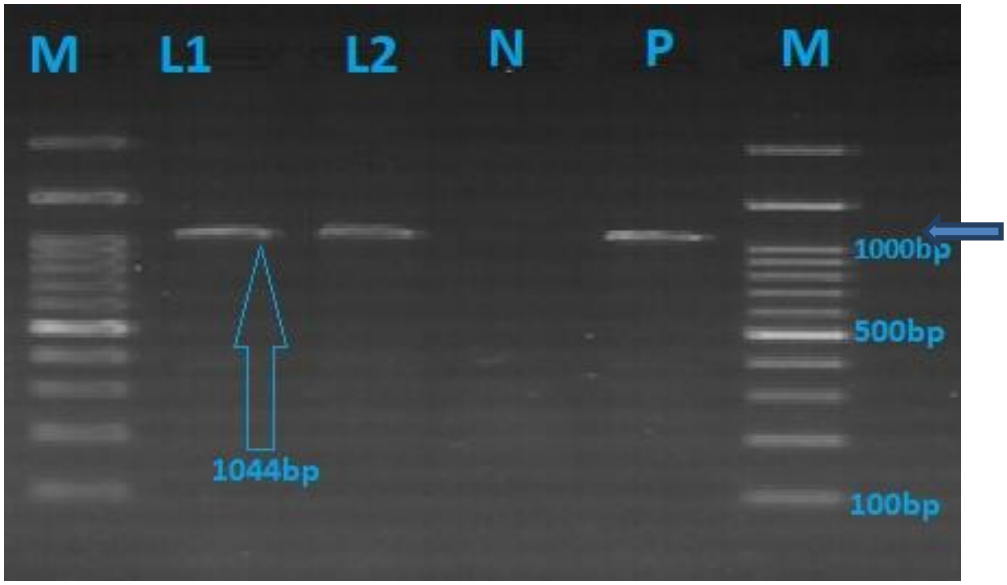


Figure 3: A conventional PCR agarose gel showing amplification of hyaD-hyaC gene with CAPA primer resulting 1044bp band size. M: Molecular ladder Biotechrabbit GmbH 100bp Berlin, Germany, Lanes: L1 and L2 are positive samples, N: Negative control, P: positive control.

4.3. Optimization of coating antigen and serum dilution

The antigen was diluted with concentration of 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, and 2 mg/ml and the positive and negative sera were diluted in the manner of 1:400, 1: 500, 1: 600, 1:800 and 1: 1000 and an indirect ELISA method was applied and evaluated. Finally 1.5 mg/ml and 1:600 were considered as the best antigen concentration and serum dilution, respectively (Figure 4).

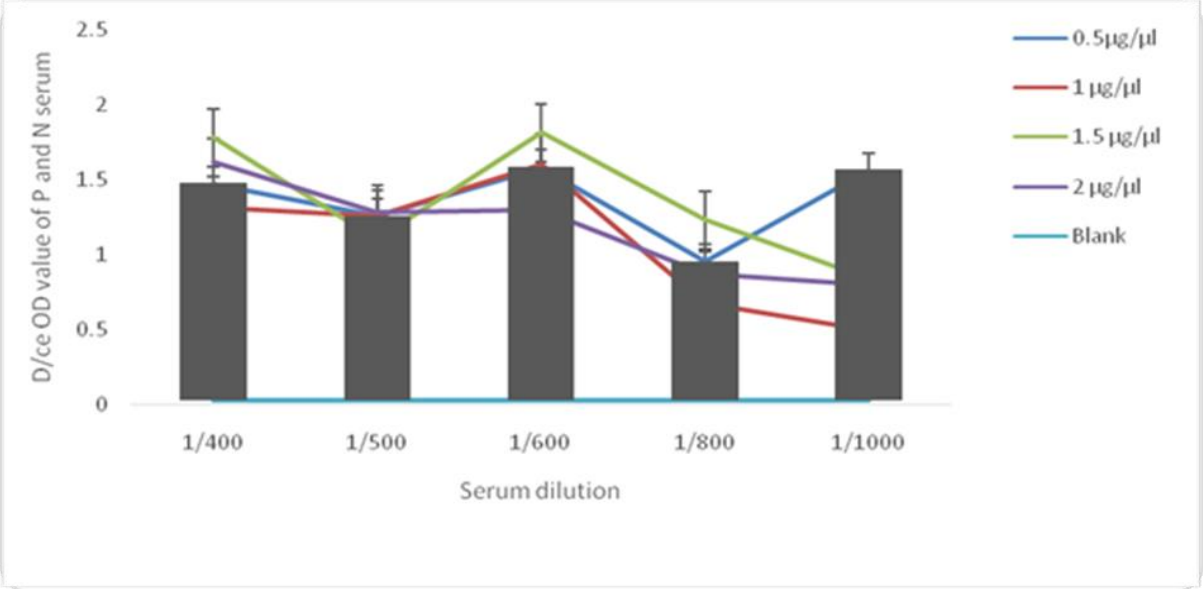


Figure 4: Bar chart showing the optimization of coating antigen and serum dilution

4.4. Optimization of Secondary antibody (Conjugate)

The secondary antibody was diluted at1:2000 and 1:4000 with different primary antibody dilution and evaluated after indirect ELISA method was applied. And 1:2000 was considered as the best working dilution (Figure 5).

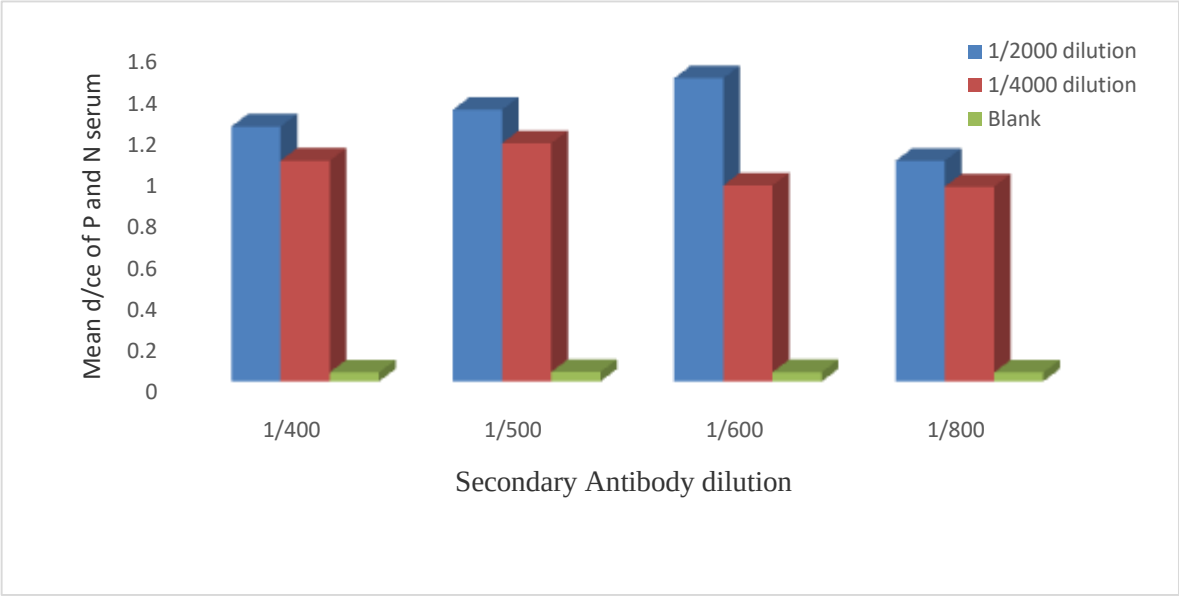


Figure 5: Bar chart illustrate secondary antibody optimization

4.5. Optimization of incubation time and temperature

For optimization of incubation time and temperature 30 minutes and 1 hour time and 25^{o}C and 37^{o}C temperature were evaluated. Finally 30 minute at 25^{o}C was selected as the optimal incubation time and temperature, respectively (Figure 6).

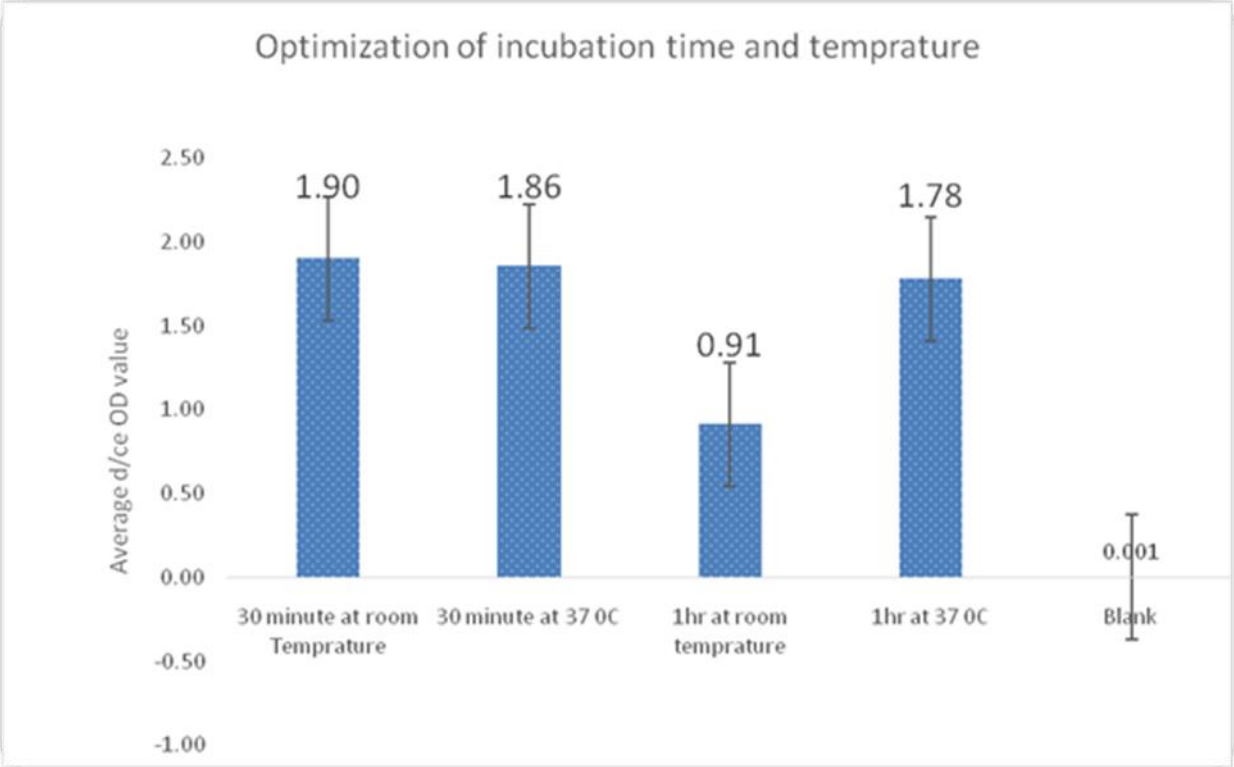


Figure 6: Bar graph displaying incubation time and temperature optimization

4.6. Determination of cut-off value

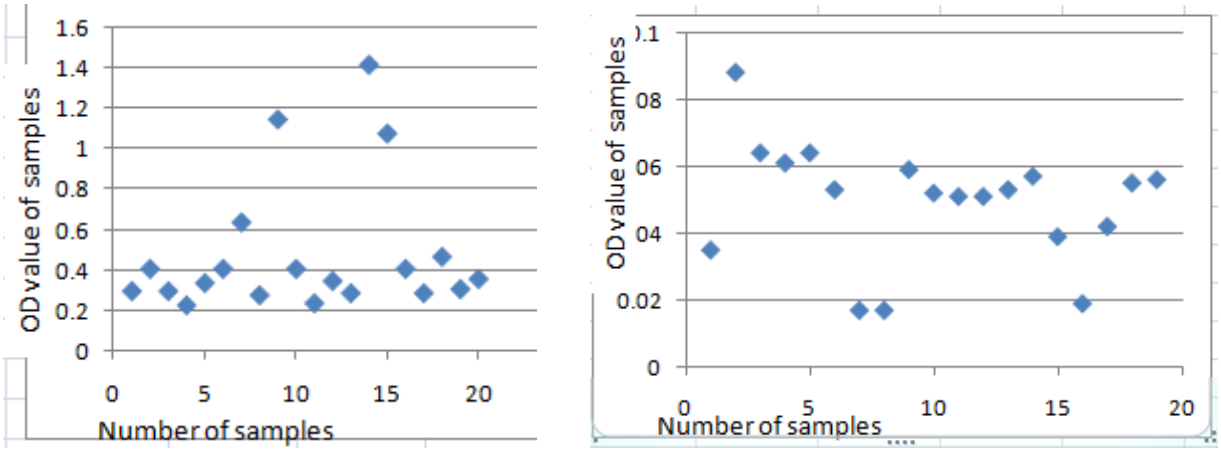
The cut-off value was determined using twenty (20) negative samples and found to be 0.28 (Table 4). Thus, the OD value greater than or equal to the cut-off value (≥0.28) was considered as positive and less than 0.28 was interpreted as negative for antibody against Fowl cholera.

Table 4: Cut-off value of the sample

List of samples	OD value	Mean	STDV	Cut off value
sample 1	0.164			
sample 2	0.161			
sample 3	0.195			
sample 4	0.106			
sample 5	0.199			
sample 6	0.144			
sample 7	0.174			
sample 8	0.177			
sample 9	0.194			
sample 10	0.135	**0.164**	**0.038**	**0.28**
sample 11	0.167			
sample 12	0.18			
sample 13	0.274			
sample 14	0.138			
sample 15	0.115			
sample 16	0.115			
sample 17	0.162			
sample 18	0.164			
sample 19	0.23			
sample 20	0.149			

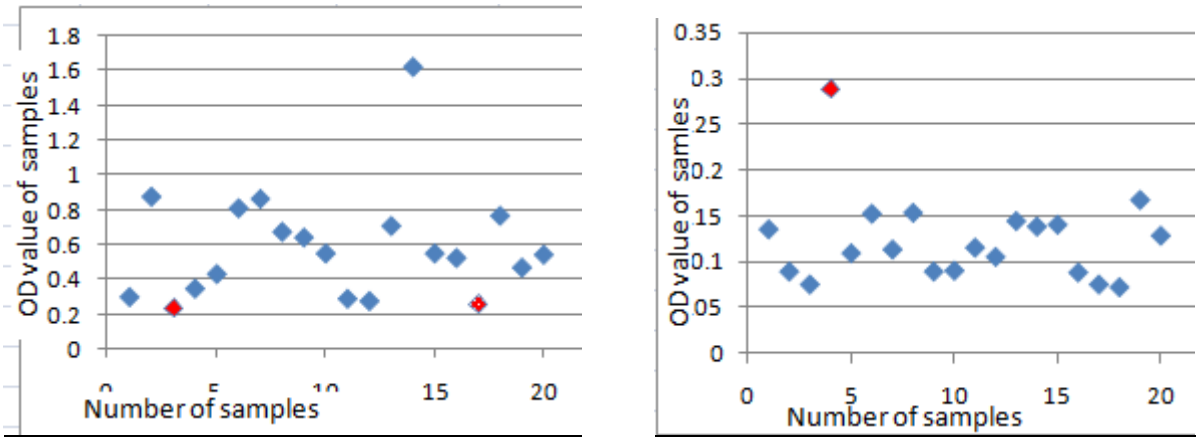
4.7. Evaluation of the test sensitivity and specificity

The method described by Samad et al. (1994) was used for the determination of the test sensitivity and diagnostic specificity by comparison with the commercial kit. As shown in figure 7; all twent (20) positive samples (a) and twenty (20) negative samples (b) were tested by commercial kit. Also as revealed in the figure 8; from twenty positive samples (a), two (2) samples were tested as negative and from twenty negative samples (b), only one (1) sample was tested as positive and the other samples tested correctely. By comparing the test results of commercial kit with newly developed In-house Indirect ELISA kit test, the specificity and sensitivity of the newly developed In-house indirect ELISA kit was 95% and 90% respectively.



a **b**

Figure 7: This scattered plot graph shows the test results of twenty (20) positive (a) and twenty (20) negative (b),total, N=40 samples after being tested by the commercial kit.



a b

Figure 8:This scattered plot graph shows the test results of twenty (20) positive (a) and twenty (20) negative (b), total, N=40 samples after being tested by the newly developed ELISA kit.

4.8. Validation of the kit

The consistency of the assay was checked by the comparison of intra and inter-assay after running the same samples within wells and between plates. As indicated on the table 5; 1.67, 8.31, 3.61 were the Percent of coefficient of variation (% CV) of sample 1, 2 and 3, respectively. The % CV of each samples were in the acceptable range and the assay was confirmed to be valid. Moreover, twenty (20) different samples were also tested on three different plates and the average % CV of all samples was evaluated, hence 7.83 was the average %CV for 20 samples (table 6).

Table 5: Validation of the kit with Intra-assay comparison between wells

List of samples	Well 1	Well 2	Well 3	Average	SD	% CV
Sample 1	0.196	0.191	0.19	0.19	0.003215	1.67
Sample 2	0.583	0.686	0.659	0.64	0.053407	8.31
Sample 3	0.556	0.557	0.592	0.57	0.020502	3.61

SD= standard deviation, % CV= Percent of coefficient of variation

Table 6: Validation of the kit with Inter-assay comparison between plates

List of Samples	Plate 1	Plate 2	Plate 3	Average	SD	% CV
Sample 1	0.329	0.352	0.35	0.34	0.012741	3.71
Sample 2	0.283	0.301	0.302	0.30	0.010693	3.62
Sample 3	0.082	0.097	0.08	0.09	0.009292	10.76
Sample 4	0.156	0.184	0.155	0.17	0.016462	9.98
Sample 5	0.185	0.206	0.167	0.19	0.019519	10.49
Sample 6	0.285	0.296	0.264	0.28	0.016258	5.77
Sample 7	0.201	0.236	0.187	0.21	0.025239	12.13
Sample 8	0.197	0.2	0.186	0.19	0.007371	3.79
Sample 9	0.11	0.13	0.128	0.12	0.011015	8.98
Sample 10	0.141	0.178	0.175	0.16	0.020551	12.48
Sample 11	0.13	0.155	0.147	0.14	0.012767	8.87
Sample 12	0.185	0.224	0.207	0.21	0.019553	9.52
Sample 13	0.177	0.2	0.221	0.20	0.022008	11.04
Sample 14	0.157	0.178	0.168	0.17	0.010504	6.26
Sample 15	0.185	0.204	0.21	0.20	0.013051	6.54
Sample 16	0.181	0.212	0.203	0.20	0.015948	8.03
Sample 17	0.128	0.137	0.115	0.13	0.01106	8.73
Sample 18	0.143	0.148	0.126	0.14	0.011533	8.30
Sample 19	0.083	0.088	0.091	0.09	0.004041	4.63
Sample 20	0.174	0.165	0.173	0.17	0.004933	2.89
Average						7.83

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5. DISCUSSIONS

The enzyme-linked Immunosorbent assay (ELISA) is a commonly used analytical biochemistry that works on the principle that specific antibodies bind with the target antigen in the sample. Earlier studies showed that ELISA assay is more effective test for detecting antibodies against Fowl cholera in avian species (Poolperm et al., 2017). This agrees with the current study which we developed a sensitive, cost-effective easily operational in-house indirect ELISA technique that can detect antibodies against Fowl cholera.

Due to its specificity to antibody detection the use of the right coating antigen onto ELISA micro plate and specific to antibody of interest is so crucial. Related studies revealed that ELISA that can detect antibodies against Fowl cholera were developed after coating the whole cell antigen, sonicated antigen, potassium thiocyanate extract, lipopolysaccharides, sodium salicylate extract, and capsular or heat extract antigen of Fowl cholera Afzal et al.(1992). In the present study, we developed ELISA based on the whole cell antigen which agree with the study described by Afzal et al.(1992). In this ELISA technique the optimal antigen concentration used to coat the plate was 1.5 mg/ml. However, it is comparably different with the findings of Poolperm et al.(2017) who carried out the heat extract antigen to coat the plate and found 1μg/ml concentration was the optimal coating antigen. Another membrane protein based antigen coating was also done by Mahboob et al.(2023), and revealed 3.13μg/μL was the best candidate for coating antigen concentration. Even when the antigen is similar or the same, the variation of optimal concentration of a coating antigen in ELISA kit development from country to country, laboratory to laboratory could be due to several reasons including methods for antigen purification or formulation, plate properties, difference in coating buffer composition and others. In general, the current analysis suggests that most studies have used various coating antigen concentrations, and the optimal coating antigen concentration is not similar in the previously stated studies to that of the current study.

The result of the test serum or antibody dilution revealed that dilution at1:600 were the optimal conditions for the newly developed ELISA assay. Thus, the result of current study is relatively in line with the commercial ELISA kit of *P.multocida* used to detect antibodies in sera of chickens and Turkey (***IDvet ID Screen®, France)***. Jung and Rautenschlein (2020), found dilution at 1:500 was the best working test sera dilution during the study of in-house

ELISA kit development for detection of antibodies against *P. multocida* in Pekin ducks. The relative similarity of these studies may be due to several interconnected factors such as similarity in antigen preparations, birds might exhibit similar immune responses to various bacterial infections and cross-reactivity in which some bacterial species might share common antigens or epitopes. This could lead to comparable antibody or test sera dilutions in the assay. Studies conducted by other researchers (Poolperm et al.2017;Tankaew et al.2017 and Mahboob et al.2023), revealed the best serum diltuion at1:100 for the development of ELISA kit for *pasteruella multocida,* which are not in agreement with the current study. This variation could be due to the difference in antigen concentration used, source of serum samples, variability in the antibody titer, and technical factors can lead to variations in the optimal serum dilution between the studies.

Secondary antibody dilutions were performed at 1:2000 and 1:4000 to find the optimal working dilutions. Thus, the ideal working dilution was found to be 1:2000. Almost similar optimal concentrations of conjugate (1:2000 dilution) was obtained for the assay with the report of Poolperm et al.(2017). In contrast, studies conducted by Aziz et al.(2022) and Sthitmatee et al.(2023) reported 1:1000 and 1:10,000 as the optimal dilution for in-house ELISA kit development, respectively. The difference could be due to the fact that variation in method of conjugate and reagent preparation, optimization procedures and others.

The best incubation temperature and time were obtained at room temperature for 30 minutes. This result was analogous with the result of Jung and Rautenschlein (2020), that revealed the suitable incubation temperature as room temperature for 30 minutes.

The cut-off value of the present in-house ELISA assay development was determined as described by Kumar and Rao (1991), using the mean absorbance of negative control at a wavelength of 450 nm (OD_{450}) plus three times the standard deviation. Accordingly, the cut-off value was 0.28. Then, the serum samples that had OD value greater than the cut-off value were interpreted as positive and value less than the cut-off value were interpreted as negative. The cut-off value obtained in this study was greater than the value determined in the study carried out by Poolperm et al.(2017) and cut-off value stated in commercial ELISA kit of *P.multocida* that used to detect antibodies in sera of chickens and Turkey (***IDvet ID Screen®,***

France). The difference of the cut-off values between the kits may be due to the methods employed to develop the kit and environment.

By comparing commercial (gold standard) and newly developed in-house indirect ELISA kits, the specificity and sensitivity of twenty (20) negative samples were calculated in this study using the methodology outlined by Samad et al. (1994). The results were 95% and 90%, respectively.

5.1. Limitations of the study

This study was limited to use only stored serum in the serum bank of NVI due to time and financial constraints to collect large number of serum samples from field to evaluate by using the newly developed in-house indirect ELISA kit method. High-speed centrifugation was utilized in this investigation to purify the antigen; hence, the facility was constrained in order to achieve effective purification.

6. CONCLUSION AND RECOMMENDATION

Fowl cholera can lead to significant economic losses for poultry farmers due to high mortality rates and reduced productivity. Quick and accurate diagnosis allows for faster interventions, minimizing financial losses. Thus, ELISA is the commonly used serological assay for the detection and screening of antibodies in the samples. The currently developed ELISA technique revealed good sensitivity, specificity, and reliability. Also, it could be concluded that, this technique can be confidentially used for the detection of antibodies against Fowl cholera in Chickens. Furthermore, using this ELISA method will be used as a source of income for NVI through selling and avoids extra costs that are frequently spent for the purchase of a commercial ELISA kit in Ethiopia.

Therefore, based on the above conclusion, the following recommendations are forwarded:

- The performance should be conducted thorough validation studies to confirm the assay's sensitivity, specificity, and repeatability using large number of field samples.
- Regarding the antigen purification method, further work should be carried out to enhance assay performance

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8. LIST OF ANNEXIS

Annex 1: Preparation of tryptose agar medium

Materials and equipments required for both tryptose agar and tryptose broth

- Incubator
- Autoclave
- Water bath
- Test tube
- Petridish
- Sensitive balance
- Laminar air flow
- Measuring cylinder
- Stove
- Flask
- Aluminum foil
- Bottle
- Denature ethanol alcohol 70%
- Reagents and biological

No Medium composition Quantity required for 1 liter

1. Tryptose ---------- 20 gm
2. Sodium chloride ---------- 5 gm
3. Agar ---------- 22 gm
4. Glucose ---------- 1 gm
5. Distilled water ---------- 1000 ml

- ✓ Sterilization by autoclaving at $121^{o}C/15$ min
- ✓ Dispense on petridish from test tube (20ml) which contain 2ml of serum and mix wisely.
- ✓ Incubate at $37^{0}c$ for over night

Annex 2: Preparation of tryptose broth medium

No	Medium composition	Quantity required for 1 liter
1.	Tryptose	20 gm
2.	Sodium chloride	5 gm
3.	Glucose	1 gm
4.	Distilled water	1000 ml

- Sterilization by autoclaving at 121^{o}C /15min
- Dispense into test tube (20ml)
- Add sterile 2ml of serum and incubate at 37^{0}c for over night

Annex 3: Fowl Cholera inoculums preparation

Materials and equipments required

- Autoclave
- Sensitive balance
- Laminar air flow
- Measuring cylinder
- Stove
- Flask
- Aluminum foil
- Bottle (Liter)
- Ethanol alcohol 70%
- Reagents and biological
- Filter apparatus
- Filter pad 0.22µm and 0.44µm (for pre-filteration)
- Funnel
- Incubator

For one liter of distilled water add:-

1. Peptone ----------10 gm
2. Di sodium Hydrogen Phosphate (Na_2HPO_4) --------- ----------3 gm
3. Sodium chloride ---------- 5 gm
4. Potassium di -Hydrogen Phosphate (KH_2PO_4) ---------- 2 gm
5. Magnesium Sulphate ($MgSO_4$) ---------- 1 gm
6. Yeast extract ---------- 2.5 gm
7. D-Glucose ----------5 gm
8. Horse serum ---------- 5 ml
9. Distilled water ---------- 1 Liter

- ✓ Adjust PH to 7.6 at 37^{o}C and sterilize by 0.22µm filter pad.
- ✓ Incubate at 37^{o}c for 72hr.

Annex 4: Protocol of Bicinchonic Acid (BCA) Protein Assay

Materials:

- ✓ BCA working reagent (see recipe below)
- ✓ BSA standard (see recipe below)
- ✓ Protein sample
- ✓ 96-well microplate
- ✓ Microplate reader

BCA Working Reagent Recipe:

- BCA reagent A: 50 mg/mL of bicinchonic acid in 2% sodium carbonate solution
- BCA reagent B: 4% cupric sulfate solution in 0.4 N sodium hydroxide
- Mix 50 parts of BCA reagent A with 1 part of BCA reagent B. Mix well and incubate at room temperature for 30 minutes before use.

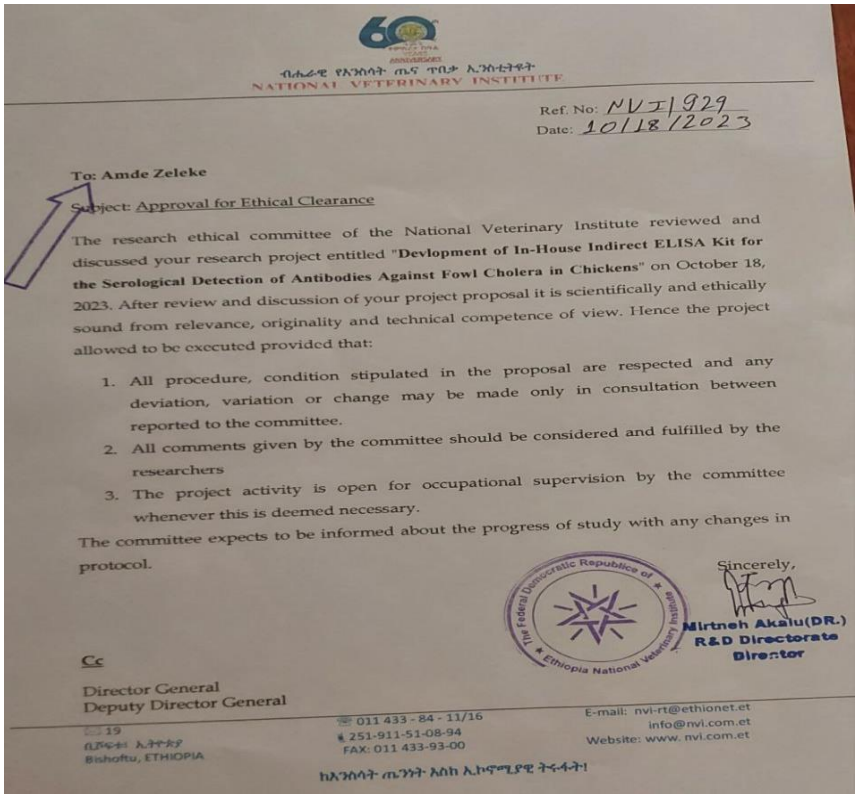
BSA Standard Recipe:

- Dissolve 10 mg of BSA in 10 mL of distilled water to make a 1 mg/mL stock solution.
- Prepare a series of BSA standard solutions with known concentrations ranging from 0 to 2 mg/mL Use distilled water to prepare the dilutions.

Procedure:

a) Prepare the BCA working reagent by mixing 50 parts of BCA reagent A and 1 part of BCA reagent B. Mix well and incubate at room temperature for 30 minutes before use.
b) Prepare a series of BSA standard solutions with known concentrations ranging from 0 to 2 mg/mL Use distilled water to prepare the dilutions.
c) Pipette 10 µL of each BSA standard solution and protein sample into separate wells of a 96-well microplate.
d) Add 200 µL of the BCA working reagent to each well.
e) Mix well by pipetting or shaking the plate.
f) Incubate the plate at 37°C for 30 minutes.
g) After incubation, cool the plate to room temperature.
h) Measure the absorbance at 562 nm using a microplate reader. It's best to read the absorbance within 30 minutes of stopping the reaction.
i) Plot a standard curve using the absorbance values of the BSA standards.
j) Calculate the protein concentration of the samples using the standard curve.

Annex 5: Approval for ethical clearance



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NATIONAL VETERINARY INSTITUTE

Ref. No: NVI/929
Date: 10/18/2023

To: Amde Zeleke

Subject: Approval for Ethical Clearance

The research ethical committee of the National Veterinary Institute reviewed and discussed your research project entitled "**Devlopment of In-House Indirect ELISA Kit for the Serological Detection of Antibodies Against Fowl Cholera in Chickens**" on October 18, 2023. After review and discussion of your project proposal it is scientifically and ethically sound from relevance, originality and technical competence of view. Hence the project allowed to be executed provided that:

1. All procedure, condition stipulated in the proposal are respected and any deviation, variation or change may be made only in consultation between reported to the committee.
2. All comments given by the committee should be considered and fulfilled by the researchers
3. The project activity is open for occupational supervision by the committee whenever this is deemed necessary.

The committee expects to be informed about the progress of study with any changes in protocol.

Sincerely,

The Federal Democratic Republic of Ethiopia National Veterinary Institute

Mirtneh Akalu(DR.)
R&D Directorate
Director

Cc

Director General
Deputy Director General

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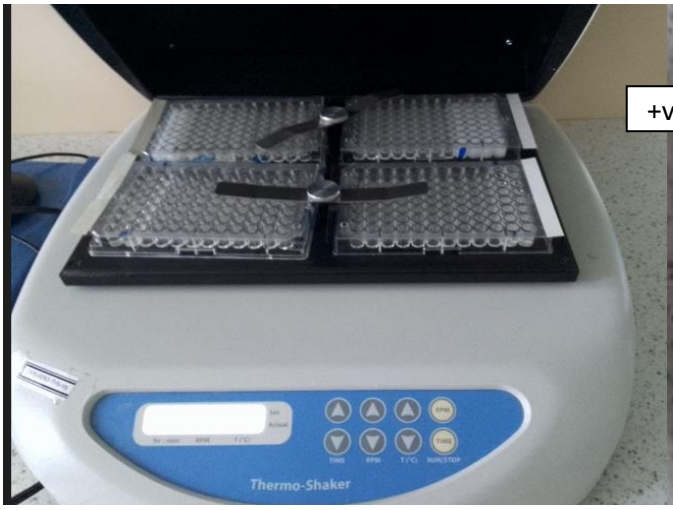
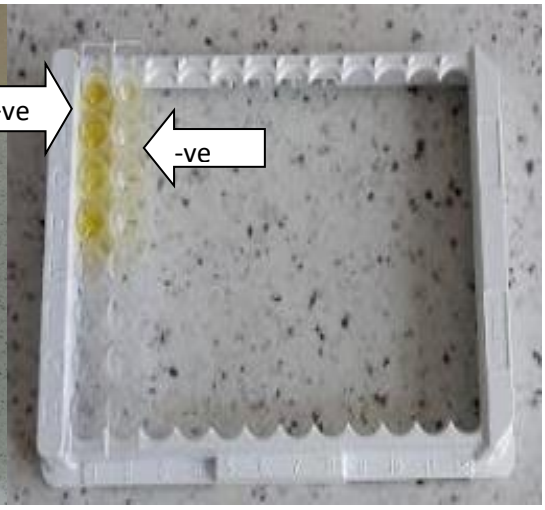
Annex 6: Miscellaneous pictures obtained during laboratory activities



Thermo shaker with ELISA plate, during agitation at 37^{o}c for 2hr.



Positive and negative samples indicated by color development and colorless respectively

Pippetting

Washing