ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

ISOLATION, PREPARATION AND EFFICACY TRIAL OF ADJUVATED FORMALIN INACTIVATED FOWL CHOLERA VACCINE FROM LOCAL ISOLATES OF *PASTEURELLA MULTOCIDA* IN LAYER CHICKENS

BY

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LIST OF ABBREVIATIONS AND ACRONYMS

AGID	Agar gel immunodiffusion test
Al $(OH)_3$	Aluminium hydroxide
Alk $(SO_4)_2$	Aluminium potassium sulphate
ANOVA	Analysis of variance
APC	Antigen presenting cells
CFU	Colony forming unit
CI	Confidence intervals
CIRAD-EMVT	Centre de- Coopération Internationale Recherche' Agronomique Pourle Development/D' Elevage et de Medecien véterinaire des po Tropicaux
CSA	Central statistical authority
DNA	Deoxy ribo nucleic acid
DSA	Dextrose starch agar
FC	Fowl cholera
FCA	Frunds complete adjuvant
GMT	Geometric mean titer
HB1	Hitchiner B1
IFA	Incomplete frunds adjuvant
IHAT	Indirect haemagglutination test
IM	Intra muscular
IR	Incidence Rate
ISA	Incomplete seepic adjuvant
LSD	Least square design
ml	Milli litter
MR	Mortality Rate
MTB	Modified tryptose broth`
NaOH	Sodium hydroxide
NB	Nutrient broth
NVI	National Veterinary Institute
OIE	Office Internationale des Epizootie

PBS	Phosphate buffered saline
PHT	Passive haemaglutination test
PI	Protective index
RBCs	Red blood cells
REA	Restriction endonuclease analysis
RPM	Revolution per minute
RR	Relative Risk
SAT	Serum agglutination test
SPSS	Statistical package for social science
TB	Tryptose broth
TFTC	Too few to count
TH2	T helper 2 cell
TMTC	Too many to count
TSB	Tryptose soy broth
USDA	United States Department of Agriculture
µl	Micro litter

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ABSTRACT

Crossectional and experimental studies were undertaken with the objectives of isolating, producing and efficacious trials of adjuvated formalin-killed fowl cholera vaccine from local isolates of *P. multocida* on layer chickens at the Department of Vaccine Production and Microbiology laboratory of the National Veterinary Institute in collaboration with Addis Ababa University, Faculty of Veterinary Medicine between September 2007 and May 2008. The study used 384 chickens having typical clinical signs of fowl cholera from commercial poultry farms and backyard poultry to detect *P. multucida.* A total of 300 Bovans Gold-line breed layer chickens were used for the experimental trial. A total of 3 local isolates of *Pasteurella multocida* (Kombolch state farm, Genesis Farm and Tadesse Farm) were identified and characterized from the natural fowl cholera out-breaks. The best growth of the isolates was in the dextrose starch agar with 5% chicken serum. All the isolates were studied for their virulence in chickens at the age of 4 weeks and found that Kombolcha State Farm isolate was highly virulent with a mortality of 100% and mortality rate of 2 chicken-day-at risk, followed by Genesis Farm isolate with a mortality of 90% and a mortality rate 1.63 chicken-day -at risk. The least virulent isolate was from Tadesse Farm, which showed no mortality. Three adjuvanted (Montanide ISA 50, $Al(OH)_3$ and $AlK(SO_4)_2$) formalin inactivated fowl cholera vaccines were prepared using three doses of bacterial loads (10^6, $10^9$and 10^{12}) from Kombolch State Farm isolate. The efficacy performances of these vaccines were evaluated and compared with each other. There were significant differences in geometric means of the antibody IHA titers between bacteria doses of 10^6, $10^9$and 10^{12}. The dose level of 10^{12} CFU elicited highest antibody response followed by 10^9 and 10^6. The three kinds of adjuvants used did not significantly differ in levels of immune responses. This study found that there was association between IHA $\log_{10}$ titers with bacteria doses (loads) and adjuvants. The linear regression indicated that the IHA $\log_{10}$ titers were associated with bacterial doses used. Multivariable correlation matrix showed very strong associations between IHA $\log_{10}$ titers and bacteria loads ($r = 0.729$). This correlation was significant. The experimental challenge protection test indicated that double doses of Aluminium potassium sulphate and Montanide ISA 50 adjuvanted vaccines gave better geometric mean antibody IHA titers and better protection (PI= 91.6% for both vaccines) as compared with aluminium hydroxide adjuvanted vaccine with PI = 83.9%. In the unvaccinated control groups 86% of mortality was recorded. This study

indicated that AlK $(SO_4)_2$ adjvanted vaccine at a bacterial dose of 10^{12} gave better IHA titers in comparison with other adjuvant and bacterial load vaccines. Although inactivated fowl cholera vaccines have been produced in other countries, the poultry enterprises in Ethiopia continue to experience problems associated with the disease due to unavailability of cost-effective vaccines. To alleviate this problem, the adaptation of this technique used in this trial with eventual production of the vaccines at the National Veterinary Institute is highly recommended.

Key words: Fowl cholera, Formalin inactivated, adjuvant vaccine, bacterial load, layers, Bovans Gold-line chickens

1. INTRODUCTION

Poultry occupies a very crucial part of our economy for being affordable, easily manageable and fast growing compared with other species of animals that provides people with animal protein. The total poultry population of Ethiopia is estimated at 56.5 million, which represents 60% of the total chicken population in East Africa. From the total population of chicken in Ethiopia, 99% are raised under the traditional backyard system of management, while 1% is under intensive management system (Tadelle *et al*., 2003; Ashenafi and Eshetu, 2004). It is quite evident that poultry farms are flourishing today but in the past mostly extensive type of production was predominating because the major part of poultry production was occupied by individual farmers and consequently, the outcome as a whole was below expectation and limited. Among the factors that played an important role in this regard are poor husbandry practices, low productive breed of the birds and various viral and bacterial avian diseases. Newcastle disease, Marek's disease, Infectious bursal disease, Fowl typhoid, Pullorum disease and Fowl cholera are the most economically important poultry diseases (Tadesse *et al.*, 2005).

Fowl cholera (FC) caused by *pasteurella multocida*, is a severe septicaemic disease of domestic and wild fowl and remains an important havoc for the poultry industry (Merchant and Packer, 1983). It usually appears as an acute, systemic disease with high morbidity and mortality, but chronic conditions with localized infections can also occur. The disease has been a subject of vast amount of research. Despite this attention, FC still remains a problem in the modern poultry industry in developed and developing countries (Merchant and Packer, 1983). In Ethiopia, even though the 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. Although it affects the birds of all ages, chickens of age less than 16 weeks are quite resistant. Mortality losses from FC usually occur in the laying flocks, because at this age birds are more susceptible than younger chickens. Capsular serogrouping and somatic serotyping accomplish antigenic characterization of *P. multocida*. Capsular serogroups are determined by a passive haemagglutination test (PHT) (Carter, 1972). Serogroups A, B, D, E, and F have been reported. But only serogroup E has not been isolated from avian hosts. Somatic serotypes are usually determined by an agar gel

immunodiffusion (AGID) test (Heddleston, 1962; Heddleston *et al.*, 1972; Arshed, 2002). Sixteen serotypes (1-16) of *P. multocida* are currently recognized. Some isolates are hybrid of two or more of these. Serotypes 1, 3, 4 and 3x4 are the most common isolates in poultry (Rhoads and Rimler, 1991). FC that occurs in young chickens is often in conjunction with some other malady and is usually caused by infection with serotype 1.

Antibacterial treatment and chemotherapy have been used extensively with varying successes. Sensitivity testing is often advantageous, since strains of *P. multocida* vary in susceptibility to chemothageous agents and resistance to treatment may develop especially during prolonged use of these agents (Rhoades and Rimler, 1991). Most producers agree that it is less expensive and economical to vaccinate fowl cholera than to treat with antibiotics. One of the best ways to be taken as prophylactic measure against fowl cholera besides good management is vaccination (OIE, 2004). Currently two types of vaccines are being used to immunize birds against fowl cholera namely, live and inactivated (Rhoades and Rimler, 1991). Even though, 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 some times 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.

Bacterins (inactivated bacterial cultures) are commercially available and have been widely used to prevent the disease. Formalin inactivated vaccines or bacterins are the best to protect animals without side effects having more advantages over the attenuated live vaccines (OIE, 2004). Bacterins are prepared from one or several serotypes of *P. multocida* that are chemically inactivated and placed in adjuvants to give high level of immunity particularly against those serotypes that are included in the vaccine. Although these vaccines have been produced in other countries, the poultry enterprises in Ethiopia have experienced problems with the disease due to unavailability of the vaccine. To alleviate this problem there has been a strong endeavors to manufacture the vaccine in National Veterinary Institute, which was the rationale for initiating the current study. So the present study was planned with the following main objectives:

1. Isolation, identification, purification and characterization of *P. multocida* from field out-break.
2. Study on factors potentiating the growth of *P. multocida.*
3. Isolation of the virulent strains (isolates) for the vaccine production
4. Preparation of adjuvanted fowl cholera vaccine.
5. Evaluating comparative efficacy of different adjuvant vaccines with respect to the difference in bacterial count.

2. LITERATURE REVIEW

2.1. The disease

Fowl cholera is a contagious bacterial disease of domesticated and wild avian species. It typically occurs as a fulminating disease with massive bacteraemia and high morbidity and mortality. 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 (Radostits *et al.,* 2000). Common synonyms for fowl cholera are avian pasteurellosis and avian haemorrhagic septicaemia. Fowl cholera is not considered to have zoonotic potential, as avian isolates are generally nonpathogenic in mammals exposed by the oral or subcutaneous routes. Other bacterial diseases, including salmonellosis, colibacillosis, and listeriosis in chickens, and pseudotuberculosis, erysipelas, and chlamydiosis in turkeys, may present with clinical signs and lesions similar to fowl cholera. Differentiation is based on isolation and identification as *P. multocida* is readily cultured from cases of fowl cholera (OIE, 2004).

2.1.1. Etiology

The etiologic agent is *Pasteurella multocida*, a gram negative, bipolar staining bacillus that grows readily on blood agar but not on macConkeys agar. Virulence among isolates is highly variable. Encapsulated strains are usually highly virulent; unencapsulated isolates are typically of low virulence. The organism varies greatly in its antigenic make up, a characteristic responsible for difficulties in producing effective bacterins and vaccines (Saville, 1995). When the gel diffusion precipitation test is used for serotyping, 16 serotypes have been identified; 13 of these from cases of fowl cholera (OIE, 2004). Serotypes 1, 3 and 3x4 are most commonly isolated from poultry out-breaks. *P. multocida* is easily destroyed by many disinfectants and by sunlight, heat and drying. Conversely, the organism persists for months in decaying carcasses and moist soil (Whiteman and Bickford, 1989).

2.1.2. Occurrence

Fowl cholera is a disease of many kinds of birds, e.g., chickens, turkeys, geese, dukes, canaries and many wild and zoologic birds. Perhaps all birds are susceptible under appropriate conditions. In poultry, most out-breaks occur in semi mature or mature birds although there are exceptions. The disease occurs more frequently in turkeys than chickens. The disease occurs frequently in domesticated waterfowl and often causes extensive lose among wild waterfowl. Geese are highly susceptible. Fowl cholera is more likely to occur in birds that are stressed by such things as poor sanitation, parasitism, malnutrition and other diseases (Radostits *et al.,* 2000). Fowl cholera occurs worldwide and is a relatively common disease. There is no relation ship between cholera in human and fowl cholera (Whiteman and Bickford, 1989).

2.1.3. Historical Information

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, Dr. Salmon studied the disease as early as 1880. Fowl cholera was one of the four major livestock diseases that stimulated formation of the veterinary division of the United States department of agriculture. Although fowl cholera has been recognized and studied for almost 200 years, it still remains an important and poorly controlled disease of poultry (Whiteman and Bickford, 1989).

2.1.4. Epizootiology

Recovered or sub clinically infected birds harbor the organism in the upper respiratory tract. Excretions, especially those from the nostrils, mouth and eyes contaminate feed and water exposing susceptible birds to infection. There is no serologic test that will detect all carrier birds. Older birds on farm can serve as a source of infection for new birds. Wild birds, including sparrows and pigeons, and many mammals, especially pigs, cat, wild rodents, reccoons, opossums, dogs and people, can disseminate *P. multocida* (Saville, 1995). The organism can persist for years in the oral cavity of rodents and carnivores. Birds bitten by such animals become

infected and disseminate the disease in the flock (Radostits *et al.,* 2000). Birds that die of septicemic cholera have the agent in most of their tissues. Cannibalism of sick or dead birds is an important method of dissemination. Scavenging of dead birds lead to oral colonization, which is an important factor for the enzootic nature of the disease. Resistance to cholera is correlated to humoral immunity. Immuno depression increases susceptibility. *P. multocida* is resistant enough to be readily spread on contaminated crates, feed bags, shoes, equipment, etc (Whiteman and Bickford, 1989).

2.1.5. Clinical Signs

With acute cholera, sudden unexpected death occurs in the flock. Mortality often increases rapidly. Laying chickens may be found dead on the nest. Geese have been reported to just drop dead while walking across a barnyard. Poisoning is often initially expected in outbreaks of acute cholera. Sick birds show anorexia, depression, cyanosis, rales, nasal and oral discharge of mucus, white watery or green mucoid diarrhea (Radostits *et al.*, 2000). The course of illness is short and often followed by death. Affected chickens often conceal themselves under equipment. Chronic fowl cholera is most common in chickens. Often there is swelling of a joint, wattle, footpad or tendon sheath. Exudates often cheesy may accumulate in a conjunctival sac or infraorbital sinus. There may be torticollis in a few birds. In turkeys with chronic cholera, abscesses in the infaorbital sinuses and torticollis are seen. In turkey breeders there is a drop in egg production and mortality following handling of during insemination. Affected toms produce thin, watery, poor quality semen (Whiteman and Bickford, 1989).

2.1.6. Lesions

Lesions may be absent if the disease is very acute. Usually there are petechial and ecchymotic hemorrhages at a few sites, e.g., on the heart, under serous membranes, in mucous membranes, on the gizzard or in abdominal fat. There is often enteritis in the upper intestine. Acute lesions develop as a result of disseminated intravascular coagulation. In layers and breeder hens free yolk in the peritoneal cavity, acute oophoritis with regressing follicles and acute diffuse peritonitis are frequently seen. These lesions can accompany many other acute diseases. In acute cases there

often is a diffuse streaking of the live. If the birds live a few days, there may be a few or many small necrotic foci in the liver ("corn meal liver"). In chronic cases there may be localized inflammatory lesions. These often involve a joint, tenden sheath, wattle, conjunctival sac, infraorbital sinus, the nasal turbinates, the middle ear or cranial bones at the base of the skull. Caseous exudates in a localized lesion should arouse suspicion of cholera. Consolidation of lungs is a common finding in affected turkeys (Radostits *et al.*, 2000). With time, these lesions become sequestered as necrotic areas in the lungs. Often these are extensive. Turkeys often have cholera as a complication of airsacculitis caused by *Mycoplasm gallisepticum*. In such cases there is usally a marked airsacculitis, pericardlitis and a well-developed fibrinous pneumonia (Whiteman and Bickford, 1989).

2.1.7. Diagnosis

At necropsy, gram-stained impression smears of liver or heart blood from septicemic cases often reveal bipolar- stained, gram- negative rods suggestive of cholera. Use of bloodstains or methylene blue readily demonstrates the bipolar morphlogy of the organism. Rabbits, hamsters or mice can be inoculated with a small quantity of exudate or minced tissue from an infected bird. These animals die within 48 hours and the organism can be isolated in pure culture from heart blood or liver. Although the history, signs and lesions may strongly suggest fowl cholera, *P. multocida* should be isolated and identified for confirmation (Radostits *et al.*, 2000). Isolates should be tested for antibiotic susceptibility because of widespread resistance and should be typed especially if routine treatment and control producers appear infective. Cholera must be differentiated carefully form erysipelas and acute colibacillosis in turkey and other birds that have both diseases. Erysipelas is caused by a gram-positive rod. Concurrent erysipelas and cholera can occur. Cholera can be differentiated readily from most septicemic and viremic diseases of poultry by culture of *P. multocida.* Cholera always should be suspected if there are epizootic losses in domesticated or wild waterfowl. Related organisms can cause cholera-like diseases or complicate other diseases including *P. gallinarum, P. haemolytica, Moraxella anatipestifer, M. osloensis*, and *yersinia pseudotuberculosis*. Several serological tests have been developed (OIE, 2004). Currently an ELISA test is commercially available and widely used. Serology is used primarily to

evaluate efficacy of vaccination rather than diagnosis of a disease out-breaks (Whiteman and Bickford, 1989).

2.1.8. Control and Prevention

Prevention

P. multocida is not transmitted through the egg. Obtain clean birds raise them in quarantine on disease free premises and away from all birds and mammals that might carriers. Never add birds to the flock, as they may be carriers. Avoid stresses, in so far as is possible, and practice a high standard of sanitation. Pike up and destroy all sick or dead birds before they can be cannibalized. Birds with cholera teem with *P. multocida* and are important in transmission of the agent (Saville, 1995). Dispose of carcasses by burying or burning to prevent them from being fed on by scavengers (including dogs and cats). Although bacterins are not always effective, in many instance they do a good job of immunizing birds, especially they can be repeated at least once. They often are given when birds are about 8 and 12 weeks old. Bacterins do not provide good cross-protection between serotypes. Oil-emulsion bacterins are used to immunize breeders prior to production. They can cause serious drops in egg production if given to laying birds (Whiteman and Bickford, 1989). Live oral vaccines for chickens and turkeys are available in many countries. In the United state live vaccines are based on the Clemson University (CU) strain of *P. multocida*. This is a naturally occurring low-virulent organism. Since its introduction as a commercial product, a slow- growing mutant has been produced (strain M-9). The latter is milder than the parent strain. They frequently are given to turkeys at 2-4 weeks intervals beginning at 6-7 weeks of age in the drinking water. Layers and breeders are inoculated by wing web stick or subcutaneously. Fowl pox vaccine can be given simultaneously. The live vaccines have been shown to be safe but problems can occur in the field presumably because of imunosupression or concurrent diseases. Live vaccines confer better resistance than killed bacterins and offer a broad spectrum of protection against most serotypes. Following an outbreak, depopulation should be considered since many surviving birds become carriers and transmit *P. multocida.* Following depopulation, the premises and equipment should be thoroughly cleaned and disinfected and, if possible, kept free of poultry for a few weeks. Continuous mediation programs have been used but are generally more costly than a vaccination program. Reduce rodents, scavengers and

predators in the farm environment and limit their contact with flocks. Differing susceptibilities among genetic lines of turkey has been shown suggesting that selection for resistance to fowl cholera may be possible (Whiteman and Bickford, 1989).

Treatment

Many sulfa drugs and antibiotics will lower the mortality from cholera but mortality may resume when treatment is discontinued. Most medications are given in the fed or water. Sulfaquinoxaline is one of the better treatments but will depress egg production in layers and may throw them completely out of production. Drugs and antibiotics in common use includes: Sulfadimethoxine, Tetracyclines, Sulfaquinoxaline, Sulfamethazine, Erythromycin, Streptomycin, Sulfamerazine and Penicillin. Addition of citric acid to drinking water and reduction of calcium or use of a chelating agent in the feed will potentiate the effects of the tetracyclines. Moving an infected flock to clean premises or markedly improving sanitation during an outbreak may slow the course of cholera. Use of live vaccine during the course of an outbreak seldom may effective. If cholera cannot be controlled, it may be necessary to market the flock early. Be sure to adhere to regulations relating to withdrawal of medication (Whiteman and Bickford, 1989).

2.2. Diagnostic techniques

Fowl cholera is a commonly occurring avian disease that can affect all types of birds and is often fatal in the peracute form (Derieux, 1978; Rimler and Glisson, 1997). Diagnosis depends on identification of the causative bacterium, *P. multocida*, following isolation from birds with signs and lesions consistent with this disease. 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.

2.2.1. Isolation and identification of the agent

P. multocida is a facultative anaerobic bacterium that grows best at 35–37°C. Primary isolation is usually accomplished by 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 butyrous. Capsulated organisms usually produce larger colonies than those of none capsulated organisms. Watery mucoid colonies, often observed with mammalian respiratory tract isolates, are very rare with avian isolates (OIE, 2004). The cells are coccobacillary or short rod-shaped, usually 0.2 – 0.4 by 0.6 – 2.5 µm in size, stain Gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found in tissue smears show bipolar staining with Wright, Giemsa stains or methylene blue, and are usually encapsulated. Isolation of the organism from visceral organs, such as liver, bone marrow, spleen, or heart blood of birds that succumb 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. The specimen is inoculated directly on to agar medium or into tryptose or another broth medium, incubated for a few hours, transferred to agar medium, and incubated again.

Identification is based primarily on the results of biochemical tests. Carbohydrate fermentation reactions are essential (Quinn *et al.,* 2002). The carbohydrates that are fermented include: glucose, mannose, galactose, fructose, and sucrose. Those not fermented include: rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, m-inositol, and salicin. Mannitol is usually fermented. Arabinose, maltose, lactose, and dextrin are usually not fermented. Variable reactions occur with xylose, trehalose, glycerol, and sorbitol. The *P. multocida* does not cause haemolysis, is non 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. Detection of hydrogen sulphide production may require lead acetate-laden paper strips suspended above a modified H_2S liquid medium (Rimler *et al.,* 1998). Commercial biochemical test kits are available. Differentiation of *P. multocida* from other avian *Pasteurella* species and *Riemerella* (*Pasteurella*) *anatipestifer* can usually be accomplished using the tests and results indicated in Table 1. Laboratory experience has shown that *P. multocida* is most easily identified by its colony morphology and appearance in Gram stain. Positive reactions to indole and ornithine decarboxylase are the most useful biochemical indications (OIE, 2004).

Table 1: Tests to differentiate *P. multocida* from other avian *Pasteurella* spps.and *R. anatipestifer*

	Pasteurella			*Riemerella*
	multocida	*M. haemolytica*	*gallinarum*	*anatipestifer*
Haemolysis on blood agar	–	+	–	v
Growth on MacConkey agar	–	+u	–	–
Indole production	+	–	–	–
Gelatin liquefaction	–	–	–	+u
Catalase production	+	+u	+	+
Urease production	–	–	–	v
Glucose fermentation	+	+	+	–
Lactose fermentation	–u	+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.

Capsular serogrouping and somatic serotyping accomplish antigenic characterization of *P. multocida.* Capsular serogroups are determined by a passive haemagglutination test (PHT)

(Carter, 1972). Serogroups A, B, D, E, and F have been reported; all but serogroup E have been isolated from avian hosts. Non-serological disk diffusion test that uses specific mucopolysaccharidases to differentiate serogroups A, D, and F have been developed (Rimler, 1994). Somatic serotypes are usually determined by an agar gel immunodiffusion (AGID) test (Heddleston, 1962; Heddleston *et al.,* 1972). Serotypes 1 to 16 have been reported; all 16 serotypes have been isolated from avian hosts (Rimler *et al.,* 1998). The most effective characterization involves determination of both serogroup and serotype. These determinations require a specialized laboratory with appropriate diagnostic reagents. To determine the serotype, the laboratory prepares the unknown bacterial culture as antigen for the AGID test and then must test it against all 16 serotype-specific antisera. Antigens present in a single isolate may react with multiple serotype-specific antisera resulting in bi- or trinomial serotypes, as illustrated by the 3, 4 and 3, 4, 12 strains (Rimler *et al.,* 1998). DNA fingerprinting of *P. multocida* by restriction endonuclease analysis (REA) has proved to be valuable in epidemiological investigations of fowl cholera in poultry flocks. Isolates of *P. multocida* having both capsular serogroup and somatic serotype in common may be distinguished by REA. Ethidium-bromide-stained agarose gels are analysed following electrophoresis of DNA digested with either Hhal or Hpall endonuclease (Wilson *et al.,* 1992; Ozobey and Muz, 2006).

2.2.2. Serological tests

Serological tests for the presence of specific antibodies are not used for diagnosis of fowl cholera. The ease of obtaining a definitive diagnosis by isolation and identification of the causative organism precludes the need for serodiagnosis. Serological tests, such as agglutination (IHA), AGID, and passive haemagglutination, have been used experimentally to demonstrate antibody against *P. multocida* in serum from avian hosts. Determinations of antibody titers using enzyme-linked immunosorbent assays have been used with varying degrees of success in attempts to monitor seroconversion in vaccinated poultry, but not for diagnosis (Arshad, 2002; Akand *et al*., 2004).

2.3. Requirements for vaccines and diagnostic biologicals

Fowl cholera may be caused by any of 16 Heddleston serotypes of *P. multocida*, although certain serotypes appear to be more often associated with the disease. The *P. multocida* vaccines in general use are bacterins, containing aluminium hydroxide or oil adjuvant, prepared from inactivated cells of serotypes selected on the basis of epidemiological information. Commercial bacterins are usually composed of serotypes 1, 3, and 4. Vaccination plays a significant role in the control of this disease. Live vaccines containing modified *P. multocida* are not generally used except in North America (OIE, 2004).

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. 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, 2004).

2.3.1. Method of manufacture

Production cultures of each bacterial isolate to be included in the final product are prepared. The cultures are typically started in small vessels and subpassaged 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).

Adjuvants have been used for more than 70 years to enhance the immune response of the host animal to an antigen. Among the mechanisms that adjuvants used to enhance the immune responses are the “depot” effect, antigen presentation, antigen targeting, immune activation/modulation, and cytotoxic lymphocyte induction (Stewart, 2000). Adjuvants such as mineral salt adjuvants, oil adjuvants, hydrophilic and hydrophobic block polymers, hydrocarbons,

surface-active agents, liposomal membranes and lipopolysacchrides have yielded promising results (Dalsgaard, 1987; Fatunmbi *et al.*, 1992). Oil-based or non-oil based adjuvants produce a vaccine depot at the inoculation site. As there is no lymphoid system in the birds (Jeurissen e*t al.*, 1988), vaccines containing adjuvant cause irritation, recruit immunocompetent cells (lymphocytes and antigen presenting cells-APC) at the injection site (Unanue, 1984). These cells phagocytose, process and present the antigen on their surface in association with self-immune associated (Ia) antigen. The thymus dependant lymphocytes (T-cells) can only recognize the antigen when presented on the surface of APC with Ia antigen (Vanio *et al.*, 1988). These antigen-stimulated T-cells transform into lymphoblasts and populate in different primary and secondary lymphoid organs such as spleen (Vanio *et al*., 1988). These cells secrete lymphokines, which potentiate the activity of bursal dependant lymphocytes (B-cells) and induce the cell-mediated immunity (Vanio and Ratcliffe, 1984).

The Montanide ISA50 (Incomplete Seppic Adjuvant) and ISA70 adjuvants are water-in-oil emulsions based on a purified mineral oil and are similar to FIA. Studies comparing these adjuvants with FCA and FIA have reported similar antibody responses but often with less inflammatory response (Johnston *et al.* 1991; Leenaars *et al.,* 1998). Other Montanide adjuvants are primarily prophylactic adjuvants that utilize a variety of mineral and metabolizable oils and are in various phases of human and veterinary trials. The surfactant for Montanide ISA 50 is mannide oleate, a major component of the surfactant in Freund's adjuvants (Stewart, 2003).

Absorption of antigens onto aluminum sulphate or aluminum hydroxide is the most common method of producing vaccines for human and veterinary medical use (Gupta and Rost, 2000). Aluminum-adjuvanted vaccines have been used in humans for more than 60 years, with an excellent safety record (Altman and Dixon, 1989). Aluminum salt adjuvants bind antigens via electrostatic forces, providing a short-lived depot effect. Aluminum adjuvants exhibit some immunostimulatory properties, and they promote almost exclusively TH_2 response to the antigen (Linblad, 2000). Granulomas consisting initially of macrophages are produced at injection sites, and there is a lymphocytic influx. Aluminum-adjuvanted antigens are rapidly cleared after injection, which leads to peak antibody titers 3 to 4 weeks after injection with a rapid decline, although repeated injections can lead to prolonged antibody responses (Altman and Dixon, 1989).

Although not extremely useful for primary immunizations for experimental antibody production, aluminum salt adjuvants have a number of desirable characteristics that warrant their consideration as adjuvants for booster injections (Stills, 1994).

2.3.2. Master seed management

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, 2004).

2.3.3. Validation as a vaccine

Efficacy

Products prepared from candidate master seeds must be shown to be effective against challenge infection. Efficacy must be demonstrated in each animal species (chickens, turkeys, ducks, psittacines) and by each route of administration for which the product will be recommended, and protection must be demonstrated against each challenge serotype for which protection is claimed. The lot of product used to demonstrate efficacy must be produced from the highest allowable passage of master seed. For live avian *Pasteurella* vaccines, 20 vaccinate 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 vaccinates must survive. The arithmetic mean count of colony-forming units in the lot of product that used to demonstrate efficacy is used as the minimum standard (immunogenicity standard). For all subsequent production lots efficacy of bacterins must be demonstrated similarly prior to licensure. However, no immunogenicity standards are derived from the lot that was used to demonstrate initial efficacy; each production lot is satisfactorily tested in a vaccination-challenge trial prior to release for sale and distribution vaccine (Islam, *et al.,* 2004; OIE, 2004).

Safety

The safety of master seeds used in the production of live vaccines must be evaluated prior to licensing. Safety must be tested in each animal species (chickens, turkeys, ducks, psittacines) for which the product is recommended. Each of 10 birds is given an equivalent of 10 vaccine doses and observed for 10 days. At least 8 of 10 birds must show no unfavorable reactions attributable to the master seed. Additionally, the master seeds must be tested for reversion to virulence and evaluated for excretion from the host and transmission to other target species (Islam, *et al.*, 2004; OIE, 2004).

2.3.4. In-process control of vaccine preparation

The purity of the cultures is determined at each stage of production prior to inactivation. This may be achieved by microscopic examination (e.g. phase–contrast microscopy, Gram strain) and/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 serials (batches) are produced in the same manner as that used to produce the serials used in immunogenicity studies (Islam, *et al.*, 2004; OIE, 2004).

2.3.5. Batch control of vaccine preparation

Sterility

Sterility tests are done on filled vaccine. Each lot must pass sterility requirements.

Safety

Safety testing is conducted on each bulk or filled vaccine lot. Bacterins are administered according to label recommendations, and the birds are observed for 14 days; at least 18 of 20 birds must show no unfavorable reactions attributable to the bacterin (OIE, 2004).

Potency

Each production lot of bacterin or live vaccine must be tested for potency by a test that is related to, and considered predictive of efficacy. Potency tests are performed on the product in its final form. Bacterins are tested for potency in a vaccination-challenge trial. Separate groups of birds (20 vaccinates, 10 controls) must be challenged with each of the serotypes of *P. multocida* for which protection is claimed. Bacterins are administered according to the dose and route recommended on the label. Two doses are administered 3 weeks apart, and all birds are challenged 2 weeks after the second dose. The birds are observed for 14 days after challenge. For a satisfactory test, at least 14 of 20 vaccinates must survive and at least 8 of 10 controls must die. The potency of live vaccine lots is determined by a bacterial count performed on reconstituted lyophilized product in its final container. The mean bacterial count of any vaccine lot at the time of preparation must be sufficiently high to ensure that at any time prior to product expiration, the count is at least twice the immunogenicity standard. The European Pharmacopoeia requires a count that is at least equal to the immunogenicity standard (Akand *et al.*, 2004; OIE, 2004).

Stability

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. Vaccines are stored at 2–7°C and protected from freezing. Partly used packs should be discarded at the end of a day's operations (OIE, 2004).

Preservatives

Any preservatives must be added within specified limits. Preservatives are generally added to vaccines to limit the growth of any contaminants introduced when the rubber cap is pierced with a needle. Ideally, multi dose vaccination equipment should be used whereby the vaccine pack is entered only once with a sterile needle (OIE, 2004).

Precautions

Vaccines prepared with aluminium-based adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate problems, but medical advice should be sought, as there is a risk of infection via a contaminated needle. Vaccines prepared with oil-based adjuvants may cause more severe reactions at the site of injection, which may manifest as large nodules. Care should be taken to administer these vaccines correctly. Operator self-injection requires immediate medical attention, involving prompt incision and irrigation of the site (OIE, 2004).

3. MATERIALS AND METHODS

3.1. Study area

The samples were collected in and around Debrezeit and Kombolcha towns where there were natural fowl cholera out-breaks. Debre Zeit is located in Oroomiya National Regional State about 45 KMs Southeast of Addis Ababa at an altitude of 1850 meters above sea level. It is found at 9^{o}N latitude and 40^{o}E longitude. It has a total human population of 95,000. This area experiences a bimodal rainfall pattern with a short rainy season from March to May and a long one from June to October. The area has an average annual rainfall of 800 mm and an average minimum and maximum temperatures of 12.3^{o}C and 27.7^{o}C respectively (CSA, 2001).

3.2. Study animals

For the identification of the isolates, the study included chickens having typical clinical signs of fowl cholera from commercial poultry farms and backyard poultry. Live chickens, sera, tissue samples or swabs were collected and submitted to the NVI Microbiology Laboratory for the isolation and identification of the bacteria.

For the vaccine trial a total of 300 day-old layer chickens purchased from Holland were used for the experiment. The chickens, which were used for the vaccine trial, were Bovans Gold line breeds from Hendrix poultry breeders, Nutreco Company, Holland. In order to avoid confounding the chickens were vaccinated for the viral and bacterial diseases. For marek's disease vaccinated at 1^{st} day of age; for first dose of Gumboro disease at 7^{th} day of age; for the first dose of Newcastel disease HB1 strain (NVI, Debre Zeit) at 14^{th} day of age; for Gumboro booster dose at 21^{st} day of age; for Newcastle booster dose of lasota strain (NVI, Debre Zeit) at 23^{rd} day of age; for fowl pox at 10^{th} week of age (NVI, Debre Zeit). To avoid confounding due to feeding all experimental groups of chickens took the same kinds of feeds and potable tap water but the feed varied with age throughout the experimental life. Starters feed, pullet's feed and layer's feed were given (Annex I and II). The chickens were conveniently classified into three groups. These

groups comprised of chickens for pathogenicity test, for vaccine efficacy trial test and for challenge protection test.

3. 3. Study design

Cross-sectional and experimental studies were undertaken. The study was under taken in two phases. The first phase was the isolation and identification of *P. multocida* from different commercial poultry farms and back yard poultry production systems that experience the natural fowl cholera out-break from September 2007 to May 2008. On the second phase, an experimental study, which included pathogenecity tests, vaccine production and vaccine efficacy trial were performed. This experiment used the local isolates (strains) collected and identified during crossectional study.

3. 4. Sampling

3.4.1. Sample size

The sample size required for this study was determined depending on the expected prevalence of *P. multucida* and the desired absolute precision. The sample size was computed using the formula given in Thrusfield (2005) as follows.

$$N = \frac{1.96^2 \, Pexp \, (1- Pexp)}{d^2}$$

Where:

N = required sample size

Pexp = expected prevalence

d = desired absolute precision

There are no previous studies on prevalence of *P. multucida* in chicken in Ethiopia. Therefore, 50% was used as the expected prevalence to estimate the sample size. Using desired 95% confidence interval, 5% precision and 50% expected prevalence the number of birds needed to

detect *P. multucida* was 384. But for the experimental study, a total of 300 layer chickens were purchased from Holland.

3.4.2. Sample collection

Attempts were made to prevent contamination and cross contamination in the course of sample collection. Thus, samples were collected in sterile containers. All samples were packed in sterile plastic bags and transported directly in cooler box with ice to the NVI Microbiology Laboratory and kept in refrigerator at 2-4°C until processed. All samples were clearly labeled. Labeling included the date of sampling, the type of sample, the farm, flock, and the hatchery (Abera, 2005). Samples including liver, spleen and heart blood were collected aseptically for isolation of the *P. multocida* from backyard poultry production system and three commercial farms in and around Debrezeit and Kombolcha State Poultry Farm, where chickens were suspected to have fowl cholera.

Collection of blood

Blood samples were collected from the wing vein of each of the experimentally vaccinated chickens without adding any anticoagulant, which is used for separation of serum to determine the antibody titer. Blood samples were collected from the chickens at pre-vaccination, two weeks of post-primary vaccination, three weeks of post-booster vaccination. Sera were separated from the blood collected without adding any anticoagulant and stored at -20 ^{o}C until tested according to Arshed (2002) and Akand *et al.* (2004).

Tissue samples

The surface of pneumonic lungs was rubbed with cotton socked in alcohol to minimize the surface contaminant. Using sterile materials this part of the lung was cut with scissors held with tongue forceps and put in another sterile screw capped universal bottles. Liver and spleen were also taken from the same carcass and put into sterile screw capped universal bottles. The universal bottles containing liver, spleen and lung samples from the same bird, were labeled with

identical numbers and the type of sample was recorded on the container. The tissues were transferred to other universal bottles containing 3ml of tryptose soy broth under sterile environment. Before transferring the samples to the broth, they were cut in slices with disinfected scissors. The scissors and tongue forceps were heated over the flame between each sample processing. Then, the samples were transported to NVI in transportation media for further processing using methods by Quinn *et al.* (2002).

3. 5. Isolation and identification of *pasteurella*

Glasswares were first soaked in detergent, washed with the same detergent solution rinsed in tap water and finally rinsed with distilled water. It was then dried and sterilized in hot oven at 160ºC for one hour. Used materials were autoclaved at 121°C for 15 to 20 minutes, cleaned from agar and other materials then soaked in detergent, washed, dried and sterilized in a hot oven. The specimen obtained from each sample by inserting a sterile platinum loop through the outer surface and inoculated into tryptic soy broth (TSB). After incubation for 24 hours at 37^0C under aerobic conditions, the cultures were examined for morphological features. All those cultures showing the presence of Gram-negative coccobicilli were further cultured on blood agar and MacConkey's agar for the preliminary identification of the isolates. Those isolates not growing on MacConkey's agar after being incubated for 5 days consecutively and those not producing haemolysis on blood agar were transferred to dextrose starch agar (DSA) and tryptic soya agar (TSA). After incubation for 24 hours at 37^0C, the cultural and morphological examinations were conducted using methods described in Collier *et al.* (1998) and Arshed (2002) (Annex III, IV, V and VI). Typical colonies with characteristic morphology of *P. multocida* were separately shifted to DSA slants in the screw caped test tubes. After incubation for 24 hours at 37^0C, the cultures were re-examined for their morphological characteristics and slants were kept under refrigeration temperature for further use as the same time the broth cultures were lyophilized (Annex IX). The purified isolates were further identified on the basis of sugar fermentation and other specific biochemical tests like indole production, methyl red, Voges proskauer, catalase, oxidase, nitrate reduction, H_2S production, urease activity, coagulase and gelatin liquefication tests. These tests were carried out according to the standard techniques described by Collier *et al.* (1998).

3. 6. Pathogenicity test

All the identified *P. multocida* isolates were examined for their pathogenicity in Bovans Gold line breed layer chickens. Fourty, 4 week-old Bovans Gold line breed layer chickens were divided into four groups (I-IV), each having 10 chickens. Chickens of group I-III received 1 ml of *P. multocida* isolates intramuscularly from Genesis Farm, Kombolcha State Farm and Tadesse Farm. The dose rate used was 10^{12}organisms. The organisms were at log (exponential) stage of growth. Group IV was the control one. The chickens of each group were observed daily for up to seven days. The incidence and mortality rates were calculated. The survivors were slaughtered and autopsies performed. Liver and heart blood were cultured for the isolation of *P. multocida*, which was a candidate for vaccine production trial. When the culture was at its log growth, it was lyophilized as a master seed bank and working seed bank. Vaccine production was carried out through the application of standard procedures for the production of inactivated bacterial vaccine using the international standards (OIE, 2004). The method of bacterial counting is given in Annex (VIII) and for the method of lyophilization in Annex (IX).

$$\text{Mortality Rate} = \frac{\text{The No of deaths due to a disease that occur in a population during a particular period of time}}{\frac{\text{The total No of population at the start of experiment + the total No of population at the End of the experiment}}{2}}$$

$$\text{Incidence Rate} = \frac{\text{The No of new cases of disease that occur in a population during a particular period of time}}{\frac{\text{The total No of population at the start of experiment + the total No of population at the End of the experiment}}{2}}$$

$$\text{Relative Risk} = \frac{\text{Incidence of disease in factor 1}}{\text{Incidence of disease in factor 2}}$$

Source: Thrusfield, (2005)

3.7. Bacterial growth studies

To obtain maximum growth of *P. multocida* different synthetic media were utilized. The inoculum size of 10^6 CFU/ml was separately inoculated into the nutrient broth (NB), tryptose soy broth (TSB), and modified tryptose broth (MTB) as described by Layton (1984). Moreover, the growth was also obtained on DSA having 5% chicken serum in Roux flask. The growth was checked after 12, 24 and 48 hours of incubation at 37^0C. Similarly another set of broth culture (TSB, TB, and MTB) was inoculated with *P. multocida* and incubated at 37^0C for two days and cultural characteristics and viable counts were recorded (Cruckshank, 1975; Arshed, 2002).

3.8. Preparation and evaluation of experimental vaccines

Three different types of inactivated experimental vaccines against fowl cholera were prepared at the Department of Vaccine Production of National Veterinary Institute of Ethiopia, following the standards given in Peters (1993) and OIE (2000).

3.8.1. Mass cultivation

As a strain for the fowl cholera vaccine production, local isolates of *P. multucida* isolated from the above experiment were used. The identified isolates of *P. multocida* were cultivated separately in Roux flasks, which contained 500 ml of fowl cholera production medium. The growth was checked through morphological examination of slide smear under the microscope and confirmed for the homogenous and pure growth of *P. multocida* (Cruckshank, 1975; Arshed, 2002).

Table 2: Fowl cholera vaccine production medium.

No	Chemical type	Unit	Quantity in litter	Quantity in vaccine	Remark
1	Peptone	Gram	10	7	
2	Casitone hydrolysate	Gram	5	3.5	
3	Sodium chloride	Gram	5	3.5	
4	Di sodium hydrogen sulphate (Na_2HPo_4)	Gram	7	4.9	
5	Potassium Di hydrogen sulphate (KH_2Po_4)	Gram	2.5	1.75	
6	Magnesium sulphate($MgSo_47H_2O$)	Gram	1	0.7	
7	Yeast extract	Gram	2.5	1.75	
8	D (+) glucose	Gram	5	3.5	
9	Horse serum	ml	10	7	10%
10	Distilled water	ml	990	693	

Source: OIE, (2000)

All the above chemicals of the media were mixed and boiled until the temperature of 70 ^oC and then the temperature decreased up to 37 ^oC to measure the pH and to add 10% serum. This was because the serum could not tolerate 70 ^oC and also the pH was dependant on temperature. The pH was measured by pH meter. Originally the pH of the medium was 6.7. But, this pH could not favor the growth of *P. multocida* organism; therefore there was a need to add 1 ml of NaOH to increase the pH to 7.4, which was ideal for the growth of the *P. multocida*. Sterilization was done by pressure filter with the pore size of 0.22 µm in the laminar flow having Bunsen burner inside in order to avoid contamination of the medium since; the medium is very suitable to most organisms. After sterilization the medium was pre-incubated at 37 ^oC for 72 hours. The total volumes of medium used for this vaccine production were 250 ml for liver isolate and 250 ml for heart blood isolate. Two vials, from liver isolate and heart blood isolate one from each of the lyophilized bacteria brought from the Micrbiology Laboratory were diluted and allowed to grow in the tryptose agar plate. After 24 hours of incubation at 37 ^oC the clear colonies were grown on the agar plate. Five to six colonies from each plate were added in two bottles of the fowl cholera vaccine production medium 250 ml each and incubated at 37 ^oC for 8 hours to find its log stage of growth. Here to determine whether the bacteria were at log growth or not the pH would be best

indicator. The pH was measured by taking 5 ml of the culture and found to be 6.3, which were indicative of the growth of the bacteria.

3.8.2. Dosage determination

This experiment aimed to know at which bacterial count (load) that this vaccine become protective to the natural fowl cholera infection. Before inactivation 1 ml of culture was titrated up to 10^{12} and the total bacteria in 1ml were counted as described by Quinn *et al.* (2002). The last titer that *P. multocida* growth laid within 30-300 CFU was at 10^{10} titer and the bacteria count found was 250 (Annex, VIII). The experiment attempted to test the protective efficacy of the vaccine at dose rate of 10^6, $10^9$and 10^{12}. To find 10^6 and 10^9 bacteria count per ml of original inactivated material, the anaculture was diluted using distilled water. For dose rate of 10^6, 1 ml of inactivated material was diluted by 10^4 ml of distilled water. For dose rate of 10^9, 1 ml of inactivated material was diluted by 10^1 ml of distilled water. The following three experimental vaccines were prepared for comparative evaluation of their efficacy under controlled experimental conditions.

3.8.3. Inactivation of the culture

There are several methods of bacterial inactivation either the protein or nucleic acid. For nucleic Acids, it is possible to use Ultraviolet light, B-propiolactone, Hydroxylamine, Oxidized spermine and Aziridine (BEI). For protein inactivation, Phenol $\pm$ Heat, Formalin $\pm$ Heat, pH and salts, Chelating agents, Detergents and Gluteraldehyde can be utilized. For the purpose of inactivation the experiment used 0.3% of formalin (40%)(OIE, 2000; Arshed, 2002). The total volume need to be inactivated was 500 ml and 0.3 % of this volume is 1.5 ml. The experiment used 1.5 ml of formalin to inactivate the whole culture. This was placed at a temperature of 37^oC for 72 hours to complete inactivation. After inactivation completed the pH was adjusted in such a way that it could not create any discomfort to the chickens going to be given. To measure the pH and other tests on inactivated material, 100ml of anaculture was taken and the pH was found to be 5.7. This was due to the addition of formalin. The pH of anaculture should be on the range of $7\pm$ 0.3 so as to avoid any discomfort after the vaccine is given to the respective chicken. The experiment used

0.17 ml of NaOH for the 100 ml of test anaculture. For the remaining of 380 ml of anaculture, 0.65 ml of NaOH was used to increase the pH to the level of 7.0.

3.8.4. Tests on inactivated material

The inactivated material after incubated at 37^{o}C for 72 hours has been tested for its sterility, safety, and purity.

Purity tests

A drop of inactivated material (anaculture) was taken and stained by Grams stain. Only gram-negative coco-bacilli *P. multocida* was found indicating that the anaculture was pure and no contamination.

Sterility test

Following incubation of the anaculture, it was tested for fungus and there was no growth on the Sabourouds agar plate indicating that the anaculture was not contaminated by fungus. On the other hand, there was also possibility that the anaculture might be contaminated with aerobic and anaerobic bacteria. To check for aerobic bacteria, a drop of anaculture was inoculated in 3 vials containing 2 ml tryptose broth each and on 3 tryptose agar plates then incubated at a temperature of 37^{0}C for 24 hours. One tryptose broth and one tryptose agar were incubated together without inoculation of the anaculture as a control. To cheek for anaerobic bacteria, the experiment was used Vian de-foie (VF) media (Annex VII). Drops of anaculture were inoculated in 3 vials of VF Media where as, one VF media was put as a control and incubated for 15 day in the anaerobic jar. After 15 days of incubation there was no growth of any anaerobic bacteria.

Safety test

Safety tests for *P. multocida* anaculture could be made on different species of animals. One ml of anaculture was given through subcutaneous route for two rabbits and kept them in cages for 15 days. Within those days there were no clinical signs observed in the rabbits.

Figure 1: a) Fowl cholera vaccine production medium. b) PH measurement

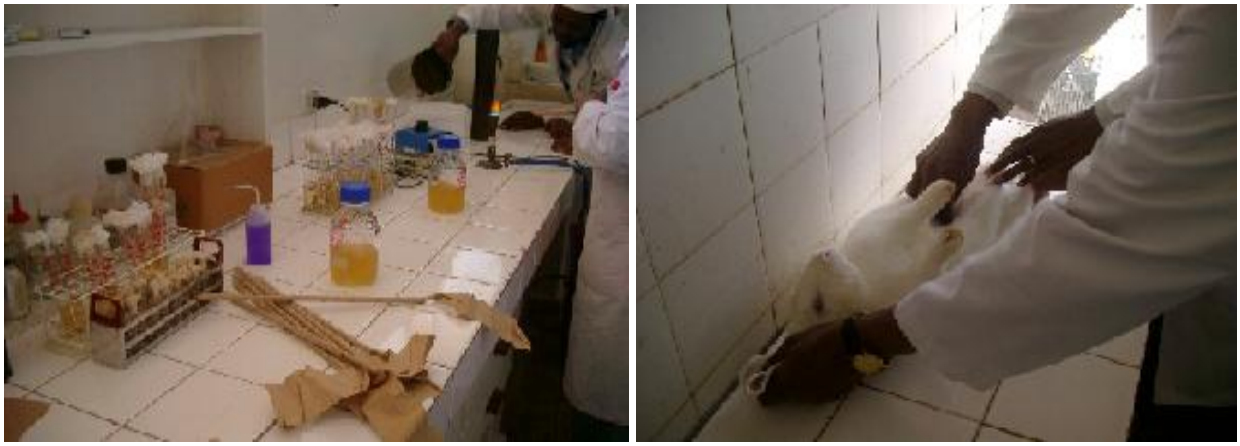


Figure 2: a) Sterility and purity test of vaccine. b) Safety test of vaccine

3.8.5. Preparation of experimental vaccines

The following three experimental vaccines were prepared for comparative evaluation of their efficacy under controlled experimental conditions. The bases for the classification of the vaccines were on bacterial counts (load) and the type of adjuvant used for the production of the vaccine. Three different kinds of adjuvants used for the production of vaccine.

1. Oil-adjuvant vaccines

Oil emulsified vaccine having Montanide ISA 50 were prepared using 50% of the culture according to the method of Stone *et al.* (1978). The Montanide incomplete Seppic adjuvants

(ISAs) (Seppic, Paris, France) are a series of adjuvants composed of a variety of oils, different emulsion characteristics, different emulsifiers, and immunomodulators. The Montanide ISA50 and ISA70 adjuvants are water-in-oil emulsions based on a purified mineral oil and are similar to FIA.

2. Aluminum hydroxide adjuvant vaccine

Aluminium hydroxide in this experiment was used 10% of the culture solution.

3. Aluminum potassium sulphate (alum) adjuvant vaccine

The ratio between the culture and the aluminum potassium sulphate was 10%.

3. 9. Evaluation of vaccine efficacy

Primary and secondary dose dependent immune responses were studied in pullets at the age of 8^{th} week by inoculating 3 dose levels (10^6, 10^9 and 10^{12} CFU/dose) of formalin-inactivated cultures of *P. multocida* isolate with deferent kinds of adjuvant.

3.9.1 Experimental design

A total of 120 pullets (8-weeks of age) were divided into 4 (A-D) groups, each having 30 pullets. Groups A-C were further subdivided into three groups of each 10 birds and each subgroup received a different dose (10^6, 10^9 and 10^{12} CFU/dose) of the isolated of *P. multocida* and different adjuvant vaccine intra muscularly, while group D was kept as control group. At day 14 post-inoculation, booster doses of each formalin-inactivated vaccines were given to the birds in groups A-C for determination of secondary immune response.

3.9.2. Vaccination of the experimental group

The experimental groups were vaccinated at 8^{th} weeks of age using 1 m1/bird of vaccine IM and the 2^{nd} vaccination was carried out 2 weeks after the first vaccination. The immunity normally develops in the next 2 weeks after vaccination (Arshed, 2002; Akand, *et al*., 2004; Islam *et al.*, 2004).

3.9.3. Collection of serum from the immunized birds

Three ml of blood samples were collected from the wing vein of all vaccinated chickens by 5 ml syringe and the serum was separated and dispensed on the micro titer plates for further use. Here strong care was taken to avoid haemolysis. Serum samples were collected at 0, 7, 14, 21, 28 and 42 day of post vaccination.

3.9.4. Determination of humeral immune response

Humoral immune response was determined by the application of serum agglutination test (SAT). This test is used only to determine whether the serum is positive for pasteurella or not. It doesn't show the level of antibody. However, the antibody titers of chickens that were immunized with the vaccine were determined by indirect haemaglutination test (IHAT) according to the method described by Tripathy *et al*. (1970); Rahman *et al.* (1994); Arshed (2002) and Akand *et al.* (2004). Blood samples were collected from the chicken at a pre-vaccination to check the presence of maternal antibody

3.10. Challenge protection test

This test was done on 60 chickens, which were 12 weeks old. From the vaccine efficacy trial, it was found that 10^{12} gave better protection than other doses. The chickens were divided into four groups (A-D) each having 15 chickens.

3.10.1. Immunization of experimental chickens

A total number of 60, Bovans Gold line layer pullets (12-weeks of age) were divided into 4 groups, A-D, each having 15 birds. Formalin inactivated *P. multocida* vaccine were inoculated IM in the birds of groups A-C, at a dose rate of 10^{12} CFU/bird but with different adjuvant. The objective was to demonstrate the protective efficacy of the produced vaccine. Group D was kept as control.

3.10.2. Challenge

Challenge cultures of the isolate were grown separately in modified tryptose broth for 8 hours at 37^0C. The challenge was done 15 days after the booster vaccination (16^{th} week age) on both vaccinated and non-vaccinated groups with 1ml of Kombolcha State Farm isolate of 10^{12} CFU/ml intramuscularly. The challenged birds were observed for a period of 15 days for the presence of clinical signs of the disease and/or mortality as described in Arshed (2002) and Islam *et al*. (2004). Mortality was recorded for a period of 14 days post-challenge. All dead birds were necropsied and their organs were cultured for *P. multocida* by streaking on DSA plates. Surviving birds were slaughtered at the end of experiment and cultured for *P. multocida*.

3.10.3. Protective Index

Since the challenge with *P. multocida* serotypes produced variable mortality in controls, the inactivated suspensions were evaluated through protective index (PI) as described by Arshed (2002).

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

3.11. Indirect haemagglutination test

Indirect haemagglutination test was performed to determine the humoral antibody production against *P. multocida* (Rahman *et al.,* 1994). It was performed in V- shaped bottom microtitration plates. This is a method in which glutaraldehyde capsular extract antigens from chicken erythrocytes, is used. The antigens were extracted by heat extraction method followed by centrifugation.

3.11.1. Materials and reagents

- Test sera of chickens
- V-shaped microplates, multichannel micropippets (Titertek ^{R}) and tips.
- *P. multocida* serotypes (CIRAD-EMVT, France).
- Positive sera (hyper immune sera) (CIRAD-EMVT, France).
- Chicken erythrocyte (NVI poultry stock)
- Phosphate buffered saline at PH 7.2 and alsever's solution.
- Gluteraldehyde 50%
- Refrigerated centrifuge (CRYOFUGE 2000-3000 rpm)
- Water bath and incubator with agitator.

3.11.2. Source of erythrocytes

Five ml of chicken RBCs were collected using a sterilized disposable syringe containing 1 ml of sterilized Alsevier solution. Erythrocytes were washed thrice with phosphate buffered saline (PBS) at 1500 rpm for 5 minutes. The packed erythrocytes, in 10 percent suspension in PBS, were used for sensitization with sonicated antigen of *P. multocida*.

3.11.3. Preparation of antigen

The reference strain of *P. multocida* was grown on dextrose starch agar enriched with 5% chicken serum for 24 hours at 37^{0}C. Cells were harvested from the solid media with 0.01M PBS

(pH 7.2) and washed three times in PBS at 2,000 rpm for 20 minutes. The sediment was resuspended in equal volumes of PBS. This suspension was heated in water bath at 60^{o}C for an hour to kill viable organisms. It was then centrifuged at 5000 rpm for 15 minutes. The clear supernatant was recovered and used as a capsular antigen extract.

3.11.4. Sensitization of erythrocytes

This was performed stepwise as follows

1. Chicken erythrocytes (from the NVI poultry farm) were freshly collected in alsever̄s solution in a ratio of 3 to 8 by volume.
2. Washed three times in PBS by centrifugation at 2000 rpm for 15 minutes.
3. 100 µl of packed RBCs were added to 10 ml of each antigen.
4. 50 µl of 50% gluteraldehyde added and homogenized by gentle shaking.
5. Incubated for one hour at 37 ^{o}C with periodical shaking.
6. Centrifuged at 2000 rpm for 10 min. and washed two times in PBS by centrifugation.
7. Finally 10 ml of PBS was added to the final sediment and a 1 % solution made.

3.11.5. Test procedure

Indirect haemagglutination antibody titers of all the serum samples were determined against *P. multocida* by microtitration technique as described by Rahman *et al.* (1994). Titerteck microtiter plates (Flow Laboratories, UK) with 8 rows and 12 columns of wells were used in the test capable of titrating 10 serum samples column-wise simultaneously. The last 2 columns (column 11and 12) of wells were left for positive and negative serum controls.

- ❖ All the wells were dispensed with 50µl PBS using multichannel microdispensor leaving the first well of each row empty in which 100µl test sera1/10 diluted in PBS were added to the first Column of the plate. Samples were placed up to column 10.
- ❖ All the serum samples were diluted by two-fold serial dilution (1:10, 1:20, and 1:40…1:320) in PBS with the help of multichannel microdispensor.

- Using multi channel pipette, 50µl of sensitized erythrocytes, was added into each well except the wells of last column of the plates, which served as negative control.
- The plates were gently tapped backward and forward and also from side to side to ensure an even suspension of erythrocytes.
- The plates were incubated at 37^0C for 30 minutes. The highest dilution of each serum sample causing a clear haemagglutination pattern was taken as end point.
- The IHA antibody titer was displayed as the reciprocal of its end point dilution. The IHA titers of all the serum samples obtained were recorded and geometrical means of titers were calculated and analyzed statistically.

3.12. Data management and analysis

The data were entered and managed in MS Excel. SPSS version 11.5 and Win Episcope 2.0 statistical softwares were applied for the data analysis. Descriptive statistics such as percentages, line plots, histograms and frequency distributions were used to describe the nature and the characteristics of data. For the pathogenicity test incidence rates, mortality rates and relative risk were calculated. Differences between groups of means were determined by one-way ANOVA test and pair wise comparisons of means. The 95% confidence intervals for the means were computed. The associations between different factors were analyzed by linear regression and bivariate correlation (Thrusfield, 2005).

4. RESULTS

Formalin inactivated adjuvanted vaccines of *P. multocida* from local isolates were prepared for the first time in Ethiopia. However, there were certain trials by individuals in NVI. Most of the techniques applied throughout the research were directly adopted from the Institute and from the (OIE, 2004).

4.1. Isolation of the organism

A total of 384 chickens suspected for fowl cholera from the backyard and intensive production systems were collected. However, all samples from the backyard chickens were negative for *P. multocida*. But, where there were out-breaks of fowl cholera in the intensive production systems all the samples were positive for *P. multocida*. Specimens of liver, spleen, heart blood and live birds collected from commercial flocks and back yard production systems where there were fowl cholera outbreaks were processed for bacterial isolation. Most (80%) of the isolated bacteria were Gram-negative coccobacilli (Figure 3) with a tendency to have bipolar staining. This property was more evident when heart blood films or impression smears of liver stained with methylene blue or Giemsa's stain were examined. In tryptic soy broth, a uniform turbidity developed and changed the color of the medium from light yellow to dirty white. On tryptic soy and dextrose starch agar, convex colonies with entire edges, mucoid and sticky nature with an approximate size of 2-3mm diameter were observed. All the isolates exhibited luxuriant on blood agar having circular, gray, smooth colonies in the form of a perfused growth. None of the isolates was able to produce haemolysis on blood agar. On MacConkey agar all the isolates failed to grow.

4. 2. Identification of the isolates

All the pure isolates that were showing typical Gram-negative coccobacilli were further processed for biochemical tests and sugar fermentation.

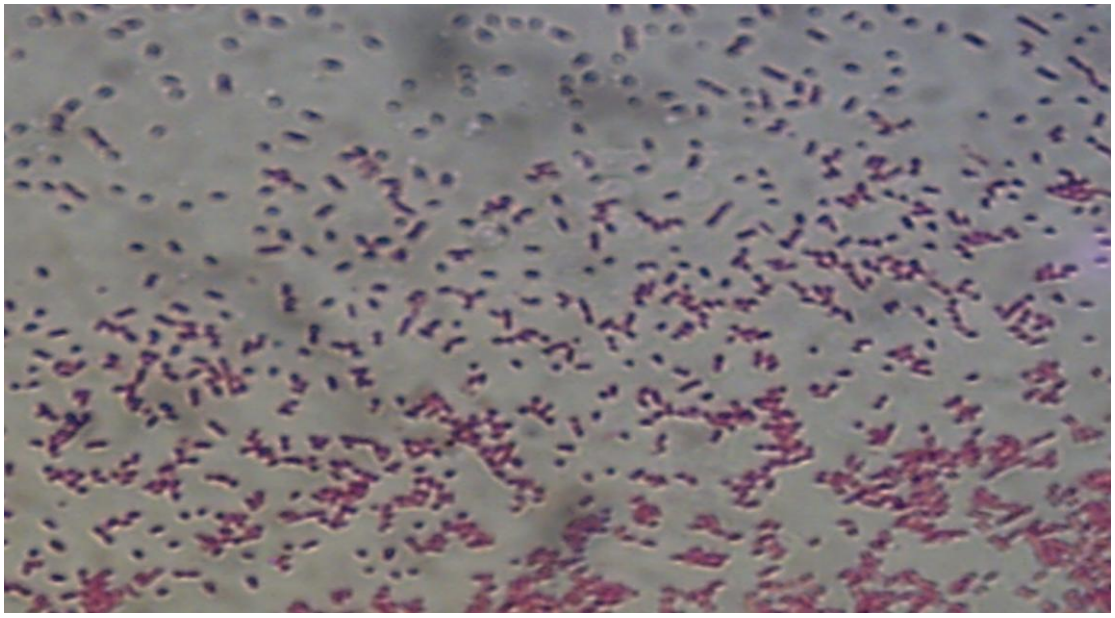


Figure 3: Gram stain smear from a culture of *P. multocida* showing gram-negative rods with a tendency towards coco bacillary forms.

4.2.1. Biochemical tests and sugar fermentation reactions

All the field isolates produced similar biochemical reactions. They showed positive reactions for catalase, oxidase, indole production, nitrate reduction and H_2S production. But, negative reactions for methyl red, Voges proskauer, urease activity, coagulase and gelatin liquefaction test. On the basis of cultural, morphological and biochemical characteristics of the isolates, *P. multocida* was isolated from the liver, spleen and heart blood samples. The sugar fermentation reactions were uniform for all the isolates. They induced fermentation of glucose, fructose, mannitol, sorbitol, mannose, sucrose and xylose with the production of acid only. A negative reaction was observed for arabinose, inositol, lactose, maltose, salicin, dulcitol and raffinose.

4.3. Pathogenicity test

Pathogenecity of the three isolates of *P. multocida* was confirmed in chickens. Kombolcha State Farm isolate was highly virulent that caused 100% mortality within 7 days of the test followed by Genesis Farm isolate 90% mortality and no mortality was recorded by the Tadesse Farm isolate (Table 3 and Figure 4a and 4b). No death was occurred in controls. The mortality rate, (2 per

chicken-day-at risk) indicated that Kombolcha State Farm isolate had high mortality rate followed by Genesis farm (1.63 per chicken-day-at risk) and no death by the Tadesse Farm isolate (0 per chicken-day-at risk)(Figure 4b). The virulent Kombolch State Farm isolate was used as a strain for the vaccine production for it was 1.22 times higher than Genesis Farm isolate.

$MR_{\text{Kombolcha isolate}} = \frac{10}{5} = 2$ per chicken-day-at risk.

$MR_{\text{Genesis farm isolate}} = \frac{9}{5.5} = 1.63$ per chicken-day-at risk

$MR_{\text{Tadesse farm isolate}} = \frac{0}{10} = 0$ per chicken-day-at risk

$IR_{\text{Kombolcha isolate}} = \frac{10}{5} = 2$ per chicken-day-at risk.

$IR_{\text{Genesis farm isolate}} = \frac{10}{5.5} = 1.81$ per chicken-day-at risk

$IR_{\text{Tadesse farm isolate}} = \frac{7}{10} = 0.7$ per chicken-day-at risk

$RR = \frac{IR_{\text{Kombolcha isolate}}}{IR_{\text{Genesis farm isolate}}} = \frac{2 \text{ per chicken-day-at risk}}{1.63 \text{ per chicken-day-at risk}} = 1.22$

Table 3: Summary results for pathogenicity test carried out on three farm isolated *P. multocida*

Day	Total challenged	Kombolcha State Farm isolate				Genesis Farm isolate (Debrezeit)				Tadesse Farm isolate (Debrezeit)			
		Morbi.	Morbid. %	Mortal	Mort. %	Morbid.	Morbid. %	Mortal	Mort. %	Morbidity	Morbid. %	Morta.	Mort. %
1	10	10	100	2	20	10	100	0	0	7	70	0	0
2	10	10	100	1	30	10	100	1	10	5	50	0	0
3	10	10	100	2	50	10	100	1	20	2	20	0	0
4	10	10	100	1	60	10	100	0	20	0	0	0	0
5	10	10	100	0	60	9	90	1	30	0	0	0	0
6	10	10	100	2	80	9	90	3	60	0	0	0	0
7	10	10	100	2	100	9	90	3	90	0	0	0	0
Total	10	10	100	10	100	10	100	9	90	7	70	0	0

Some of the acute cases showed signs of classical fowl cholera within 24 hours with mucoid droppings and viscous nasal and oral discharges, but general dullness and inappetance were the main signs in sick birds. When deaths occurred postmortem lesions of septicemic nature were found. In some of these cases there were haemorrhagic and pinhead necrotic foci in the liver (Figure 5a) and pericarditis. An active inflammatory reaction at the injection site was present. In the survivors, lesions were mainly in the muscles around the inoculation sites. Overt lameness in survivor bird inoculated with the Genesis Farm isolate was evident. Slight to severe erosive lesions with swelling of adjacent tissues, discolorations of muscle, patchy hyperemia, petechation and fibrinous exudates on the muscle surfaces and between muscle groups were observed. Air-sacculitis and sternal bursitis were also observed in some survivors.

P. multocida was isolated from liver and heart blood of all the acutely died birds (Figure 5b). The kombolcha isolate was cheeked for purity and sterility by Gram's stain and then cultured in the lyophilizer media. The culture was divided into several 1 ml vials and kept in the lyophilizer machine. The vials were put in such a way that permits the out flow of moisture through the process of sublimation (Annex IX). After 24 hours of lyophilization, two vials of kombolcha isolate were submitted to Department of Vaccine Production of NVI from Microbiology Laboratory of NVI.

Figure 4: a) Chickens inoculated with a Kombolcha State Farm isolate. b) Chickens inoculated with Tadesse Farm isolate.

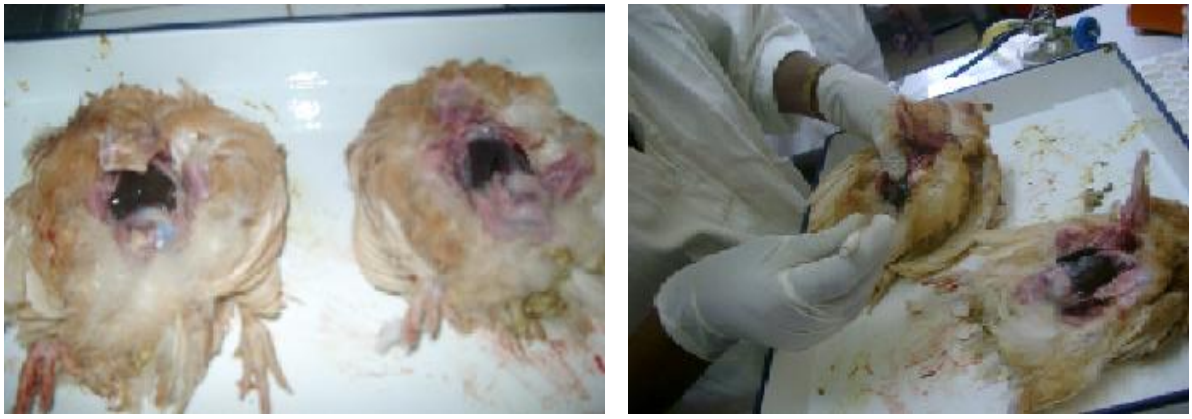


Figure 5: a) Hemorrhagic liver during autopsy. b) Sample taking for bacterial isolation.

4.4. Bacterial growth studies

Bacterial growth studies of *P. multocida*, isolated from field outbreaks of fowl cholera were conducted by culturing the organism in nutrient broth (NB), tryptic soy broth (TSB) and modified tryptose broth (MTB) at 37^0C for 12, 24 or 48 hours of incubation. A poor growth was observed in flasks of NB. Moderate growth occurred in stationary incubated flasks of TSB and MTB. Shaking produced heavier growth. The best viable count of *P. multocida* was obtained when the organism was cultured in MTB and incubated at 37^0C for 24 hours. An excellent growth was obtained in Roux flask having DSA with 5% chicken serum after 24 hours incubation at 37^0C. A viable count of 10^{12} CFU/ml was recorded.

4.5. Preparation and evaluation of fowl cholera vaccines

4.5.1. Oil-adjuvant vaccines

Oil emulsified vaccine having Montanide ISA 50 (Seppic, Paris, France) were prepared as culture to oil ratio 1:1 according to the method of Stone *et al.* (1978). Three types of vaccine having oil adjuvant depending on bacterial count were produced for this experiment. Those were vaccines having *P. multocida* load of 10^6, 10^9 and 10^{12} CFU/ml.

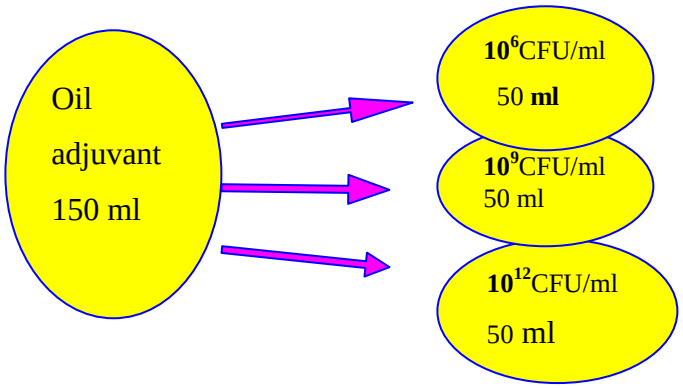


Figure 6: Schematic diagram that shows the produced oil adjuvanted vaccine.

4.5.2. Aluminum hydroxide adjuvant vaccine

Aluminum hydroxide 3 grams, that is white powder was mixed with distilled water to form 10 % solution. The adjuvant was added as 10% of the culture so that 90 ml of vaccine was prepared which was 30 ml for each bacterial lode to test. There was 10 mg of aluminum hydroxide powder per ml of the vaccine.

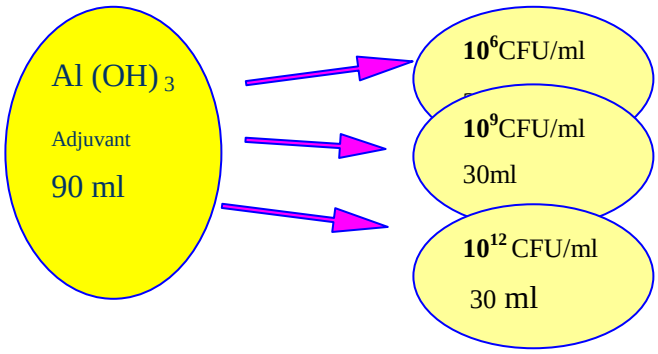


Figure 7: Schematic diagram that shows the produced Al (OH) $_3$ adjuvanted vaccine.

4.5.3. Aluminum potassium sulphate (Alum) adjuvant vaccine

Aluminum potassium sulphate (Alum) 3 grams, that is white powder was mixed with distilled water to form 10 % solution. The adjuvant was added as 10% of the culture so 90 ml of vaccine was prepared which is 30 ml for each bacterial lode to test. There was 10 mg of Aluminum

potassium sulphate (Alum) powder per ml of the vaccine. The schematic diagram that shows the produced Alk $(SO_4)_2$ adjuvanted vaccine is the same with Al $(OH)_3$.

4. 6. Evaluation of fowl cholera vaccines under controlled conditions.

Before doing any data analysis, it is worth enough to know the nature and distribution of the data. The data of this experiment was skewed to the right. For the skewed data, it was crucial to transform to normal distribution so as to use parametric statistical tests. The best method of data transformation that fitted for the data that was right skewed and the test carried on serology was logarithmic transformation. The data was normalized by $\log_{10}$ transformation (Figure 8 a and b) (Chap, 2003).

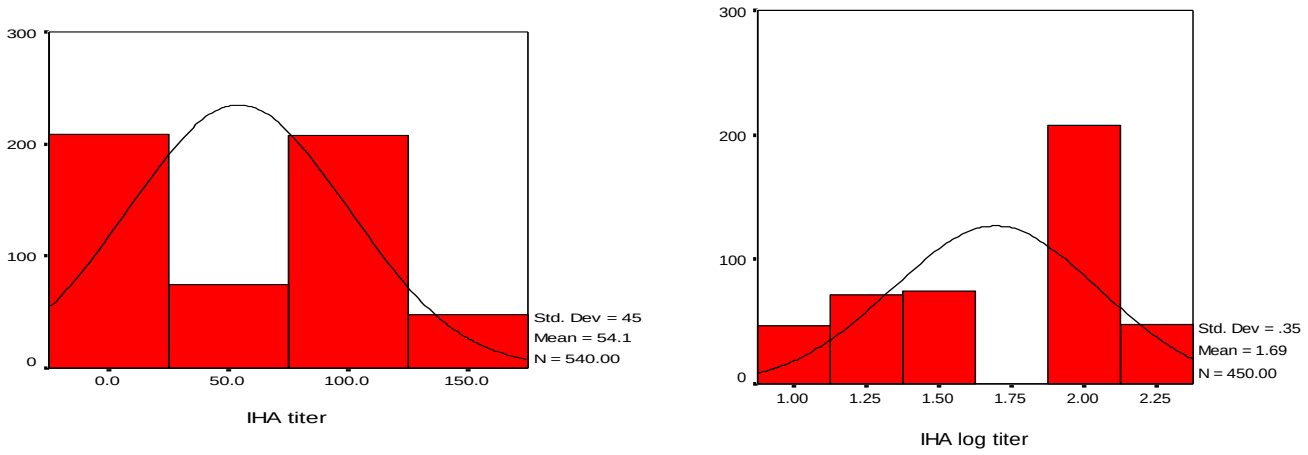


Figure 8: a) Data before transformation which is right skewed. b) Transformed data, normally distributed.

All the groups of pullets were found serologically negative for *P. multocida* antibodies at day 0. Mean IHA antibody titers increased significantly from day 7 to day 14 and declined on day 21 post-vaccination. However, the pullets that received booster dose at day 14 post- priming, the antibody titers rose significantly 7th day post-boosting ($P<0.05$) (Figure 9 and Annex XI).

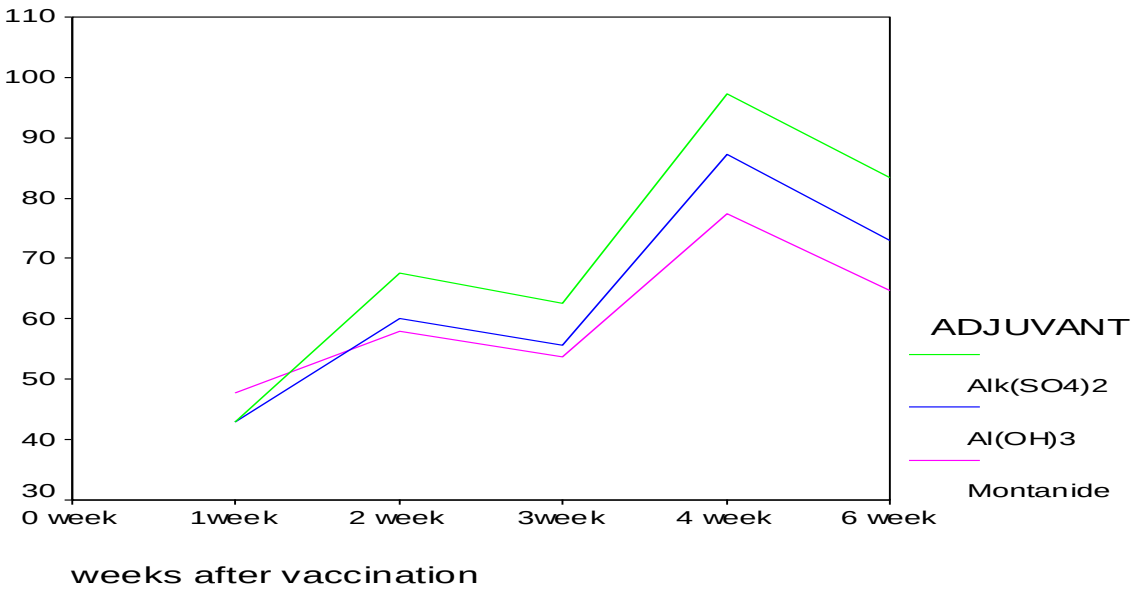


Figure 9: The antibody titer dynamics in the six weeks of vaccination.

Table 4: The geometric means of the antibody titers by dose levels and days post inoculation.

Groups	Dose level (CFU)	Days post inoculation		
		7	14	21
Aluminium potassium sulphate vaccine	10^6	13	23	20
	10^9	45	84	80
	10^{12}	64	96	88
Aluminium hydroxide vaccine	10^6	12	20	19
	10^9	49	80	72
	10^{12}	68	80	76
Montanide ISA50	10^6	11	18	17
	10^9	68	76	72
	10^{12}	64	80	72
Control		0	0	0

Dose level of 10^6 CFU elicited least antibody titers in all post inoculation days compared to dose levels of 10^9 and 10^{12} CFU. Overall, significant differences (P = 0.00, Table 5) among these dose levels were observed. Pair wise comparisons showed significant differences (P = 0.024, Table 6)

between the mean antibody titers by dose levels. However, no differences (P = 0.273, Table 5) were observed among mean titers by the types of adjuvants.

Table 5: The ANOVA on bacteria load and adjuvant to see mean differences of IHA log titers.

Source of variation	Difference	Sum of Squares	df	Mean Square	F-test	P-value
Bacteria load	Between Groups	36.484	2	18.242	418.40	0.000
	Within Groups	19.489	447	0.044		
	Total	55.974	449			
Adjuvant	Between Groups	0.324	2	0.162	1.300	0.273
	Within Groups	55.650	447	0.124		
	Total	55.974	449			

df = degrees of freedom

Table 6: Summary results of pair wise comparisons of dose levels and adjuvants on IHA log titers.

Log titer	Variable constant	Variable compared	Mean difference	Std. error	P-value	95% C.I.	
						Lower Bound	Upper Bound
Bacteria load	10^6	10^9	-.57(*)	.024	.000	-.62	-.53
		10^{12}	-.63(*)	.024	.000	-.68	-.58
	10^9	10^6	.57(*)	.024	.000	.53	.62
		10^{12}	-.06(*)	.024	.020	-.10	-.01
	10^{12}	10^6	.63(*)	.024	.000	.58	.68
		10^9	.06(*)	.024	.020	.01	.10
Adjuvant	Alk $(SO_4)_2$	Al $(OH)_3$	.04	.041	.279	-.04	.12
		Montanide	.06	.041	.116	-.02	.14
	Al $(OH)_3$	Alk $(SO_4)_2$	-.04	.041	.279	-.12	.04
		Montanide	.02	.041	.623	-.06	.10
	Montanide	Alk $(SO_4)_2$	-.06	.041	.116	-.14	.02
		Al $(OH)_3$	-.02	.041	.623	-.10	.06

* The mean difference is significant at the .05 level.

CI = confidence intervals

It is also worth enough to see the impact of bacterial load on log titer of IHA at different kinds of adjuvant. IHA titer stimulated by each bacterial load was highly significant by all three kinds of adjuvants (Table 7). But, there was no significant difference ($P > 0.05$) on log titer stimulated by 10^9 and 10^{12} bacterial loads at each specific adjuvant (Table 7). The two bacterial loads stimulate nearly the same amounts of IHA log titer.

Table 7 : Pair wise comparision of bacterial load on IHA log titer at different kinds of adjuvant.

Adjuvant	Constant Bacterial load	Comparable Bacterial load	Mean difference	Std. error	P-value	95% C.I.	
						Lower Bound	Upper Bound
Alk $(SO_4)_2$	10^6	10^9	-.57(*)	.043	.000	-.65	-.48
		10^{12}	-.63(*)	.043	.000	-.72	-.55
	10^9	10^6	.57(*)	.043	.000	.48	.65
		10^{12}	-.07	.043	.122	-.15	.02
	10^{12}	10^6	.63(*)	.043	.000	.55	.72
		10^9	.07	.043	.122	-.02	.15
Al $(OH)_3$	10^6	10^9	-.54(*)	.047	.000	-.63	-.44
		10^{12}	-.60(*)	.047	.000	-.70	-.51
	10^9	10^6	.54(*)	.047	.000	.44	.63
		10^{12}	-.07	.047	.162	-.16	.03
	10^{12}	10^6	.60(*)	.047	.000	.51	.70
		10^9	.07	.047	.162	-.03	.16
Montanide	10^6	10^9	-.62(*)	.034	.000	-.69	-.55
		10^{12}	-.66(*)	.034	.000	-.72	-.59
ISA 50	10^9	10^6	.62(*)	.034	.000	.55	.69
		10^{12}	-.04	.034	.290	-.10	.03
	10^{12}	10^6	.66(*)	.034	.000	.59	.72
		109	.04	.034	.290	-.03	.10

* The mean difference is significant at the .05 level.

The above statistical tests were used to see the significant differences of log titers at different kinds of independent variable like bacterial load and adjuvants. But, the linear regression indicated that the IHA log titer was associated with bacterial load (P = 0.00, Table 9). On the other hand multivariable correlation showed strong association between log titer and bacteria load (r = 0.729). The correlation was significant at 99 % CI (Table 11).

Table 8: The linear regression between bacterial load and adjuvant with IHA log titer

Predictors: (Constant)	Dependent Variable	Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
Bacteria load	Log titer	1	0.72	0.53	0.531	0.24
Adjuvant	Log titer	1	0.07	0.006	0.003	0.35

Table 9: ANOVA on linear regression of bacterial load and adjuvant with IHA log titer.

Predictors (Constant)	Dependent Variable		Sum of Squares	df	Mean Square	F-test	P-value
Bacteria load	Log titer	Regression	29.782	1	29.7	509.4	0.000
		Residual	26.191	448	0.05		
		Total	55.974	449			
Adjuvant	Log titer	Regression	.309	1	0.30	2.48	0.115
		Residual	55.664	448	0.12		
		Total	55.974	449			

Table10: The linear regression coefficients between bacterial load and adjuvant with IHA log titers.

Model	Variables	Un standardized Coefficients		Standardized Coefficients	t-test	P-value
		Beta	Std. Error	Beta		
1	Constant	0.119	0.071		1.68	0.09
	Bacterial load	0.315	0.014	0.729	22.5	0.00
2	Constant	1.759	0.044		40.00	0.00
	Adjuvant	-0.032	0.020	-0.074	-1.57	0.115

The association between the adjuvant and IHA log titer was assessed using regression and correlation analysis. The beta coefficients were very positive for bacterial loads and significant ($P < 0.05$, Table 10) and negative for adjuvants. However, they were very low (less than 1). The correlation matrix (Table 11) showed significant positive Pearson's correlation coefficients between log titer and bacterial load ($r = 0.729$, $P = 0.00$) and bacterial load and adjuvant ($r = 0.657$, $P = 0.00$)

Table 11: The correlation matrix between bacterial loads, adjuvant types and IHA log titers.

		Log. Titer	Bacterial load	Adjuvant
Log. Titer	Pearson correlation coefficient	1	0.729(**)	-0.074
	P-value	.	0.00	0.115
Bacterial load	Pearson correlation coefficient	0.729(**)	1	0.65(**)
	P-value	0.00	.	0.00
Adjuvant	Pearson correlation coefficient	-0.074	0.65(**)	1
	P-value	0.115	0.00	.

** Correlation is significant at the 0.01 levels.

4.7. Challenge protection test

The vaccine efficacy trial showed that 10^{12} was better in stimulating antibody production in chickens accordingly; this dose was selected as a candidate for the challenge protection test. The results of this test are given in Table 4 and 6.

Survival of the birds that received booster dose of fowl cholera vaccine in aluminum hydroxide adjuvant at a dose of 10^{12} was 86.7 % against *P. multocida* isolate challenged at 16th week of age (two weeks of post booster vaccination) (Table 12). The birds primed and boostered with aluminum potassium sulphate (alum) showed 93.4% survival against *P. multocida* isolate challenge at 16th week of age (two weeks of post boostering). On the other side, 93.4% birds that took montanide ISA 50 adjuvanted vaccine survived from the challenge. The control groups, which were at the same age but did not take the vaccine and challenged with the *P. multocida* isolate. Hence, there was mortality of 86%. The protective index was the best parameter to measure the protective ability of the vaccine. Good, effective vaccines should have a PI of at least 80%. The protective indices recorded for aluminium hydroxide $Al(OH)_3$, aluminium potassium sulphate $AlK(SO_4)_2$ and montanide ISA adjuvanted vaccines were 83.9 %, 91.6 % and 91.6 % respectively.

4.7.1. Signs and lesions

Mortality in the unvaccinated birds was 86% (Table 12). Birds started dying within 24 hours of challenge without showing perceptible signs. The necropsy findings in the dead birds consisted of epicardial petechae and diffuse subserosal haemorrhages in the parietal and visceral peritoneum. An increased peritoneal fluid was frequently noted. In most of the cases the liver tissues were markedly enlarged, although few had focal necrosis. Spleens were invariably swollen and hyperemic and had focal hemorrhages. In addition, the dead birds generally had extensive swelling of the kidneys. After 7th day post-challenge, survivors showed neither of the above lesions. The cultures from heart blood, liver and lungs showed typical non-haemolytic growth of Gram-negative coccobacilli that later identified as *P. multocida*. All the samples taken from the survivors were negative for *P. multocida*.

Table 12: Challenge protection test to different adjuvanted vaccines.

Day	Control		$Al(OH)_3$		$AlK(SO_4)_2$		Montanide ISA 50	
	Died	Sick	Died	Sick	Died	Sick	Died	Sick
1	-	-	-	-	-	-	-	-
2	1	12	0	3	0	0	0	0
3	2	12	0	3	0	0	0	0
4	5	8	0	3	0	0	0	0
6	8	5	0	3	0	0	0	0
8	9	4	0	3	0	0	0	0
12	12	1	0	3	0	1	0	2
14	13	0	0	2	0	1	0	2
15	13	0	2	1	1	0	1	1
Mortality	86%		13.3%		6.6%		6.6%	

5. DISCUSSION

Fowl cholera is a bacterial disease of domestic poultry and other fowl. This disease has resulted in substantial economic losses to the poultry industry due to high mortality rate, loss of egg production, condemnations and medication costs.

In this study, attempts were conducted for the isolation of *P. multocida* from natural outbreaks of fowl cholera from backyard and intensive poultry production systems in and around Debre zeit and Kombolcha State Farm. All the isolates were examined for their cultural, morphological and biochemical characteristics. None of the isolates was able to grow on MacConkey's agar. The colony characteristics were best studied on blood agar, where the growth was perfused with small grayish colonies without any evidence of haemolysis. Similar results have been reported (Heddleston *et al*., 1964; Rhoades and Rimler, 1991; Divivedi and Sodhi, 1998; Arshed, 2002). Colonial morphology observed with obliquely transmitted light is one of the most useful characteristics in the study of *P. multocida* (Rhoades and Rimler, 1991; Arshed, 2002). In this study, circular, smooth, convex, translucent, iridescent and glistening colonies were observed on dextrose starch agar after incubation at 37^0C for 24 hours. These results are in line with those of Collier *et al*. (1998) and Arshed (2002). Hanging drop method was used to study the motility of the organism in respect of all the purified isolates and was found non-motile. Results of sugar fermentation and other biochemical tests were also in line with the findings of (Crickshank, 1975; Liowa and Toe, 1978; Rhoades and Rimler, 1991).

5.1. Pathogenicity

Pathogenicity of all the *P. multocida* isolates was confirmed in chickens. In the present study, two of the three isolates were highly virulent with 100% mortility by Kombolcha State Farm isolate and 90% by Genesis Farm isolate. But no mortality was recorded in birds that receive Tadesse Farm isolate. The mortality rate and incidence rates were calculated. The mortality rate from Kombolcha State Farm, Genesis Farm and Tadesse Farm isolates were 2.0,1.63 and 0 per-chicken-day at risk respectively. The incidence rates were 2.0,1.81and 0.7 per- chicken-day at risk respectively. This test indicated that the Kombolch State Farm isolate was highly virulent

(RR= 1.22) than Genesis Farm and Tadesse Farm isolates. Kombolcha State Farm isolate was chosen for the vaccine production. These findings are in agreement with the results of Rimler (1987) who reported that *P. multocida* strains varied in virulence. Rimler (1987) reported that strains representing serotypes 1, 3 and 4 were the most virulent. Arshed (2002) isolated and serotyped a total of 21 isolates. Arshed (2002) found that strains 1 and 3 were highly virulent with 100 % and 80 % mortality respectively. Curtis *et al.* (1980) reported a considerable variation in the virulence of isolates of *P. multocida* from British avian sources, for mice, chickens and turkey poults. Rhoades and Rimler (1991) exposed turkey poults intraphrygneally and intravenously to two antigenically and biochemically similar strains. Based on mortality, strain P-1059 was highly virulent and strain P-1062 was avirulent. Schmimmel and Van (1995) checked the virulence of *P. multocida* in the embroyonated eggs and reported that the organism caused death in the embryonated eggs. Matsumoto and Strain (1993) found that both capsulate and non-capsulate forms of *P. multocida* apparently increased their pathogenicity by bird-to-bird transmission in a short period. Virulence of *P. multocida* strains can be variable as well as complex. The degree of virulence has been reported to vary depending on the host, the bacterial strain and the environmental interactions (Hansen and Hirsh, 1989; Christensen and Bisgaard, 2000; Arshed, 2002). The capsule was shown to be the virulence factor for *P. multocida* serogroup A in mice as well as in chicken (Chung *et al*., 2001).

The results of bacterial growth studies revealed that the optimum growth of *P. miltocida* could be obtained by culturing the organism in modified tryptose both having 5% chicken serum and incubated at 37^0C for 24 hours in shaking culture condition. These findings are in line with the results of Layton (1984) and Arshed (2002) who produced dense growth of *P. multocida* in modified tryptose broth (MTB) when the media was continuously shaken or aerated. The media contain carbohydrate and increased amounts of nitrogen. *P. anatipestifer* behaved similarly when grown under similar conditions (Layton and Sandhu, 1984). An excellent growth was also observed on dextrose starch agar. Rhoades and Rimler (1991) and Arshed (2002) also reported that dextrose starch agar with 5% avian serum is an excellent medium for isolation and growth of *P. multocida.* Moreover, the growth is enhanced when the medium is enriched with peptone, casine hydrolysate, or avian serum. Chung *et al.* (2001) obtained an excellent growth of *P. multocida* strains on dextrose starch agar supplemented with 6% chicken serum. Bain *et al*.

(1982) reported that the sugar content in the medium was responsible for enhancing the capsule production.

5.2. Vaccine efficacy trials and challenge protection test

Fowl cholera vaccine production medium was utilized for the mass cultivation of *P. multocida* at 37^0C for 24 hours of incubation. The growth was checked through morphological examination of slide smear under the microscope and confirmed for the homogenous and pure growth of *P. multocida*. The bacterial count was adjusted so that 1ml of each suspension contained 2.5 x 10^{12} CFU. This is nearly the same with the work of Heddleston, and Reisinger (1960) who grew and harvested culture of *P. multocida* at the bacterial concentration adjusted to 25 x McFarland 1 density (approximately 3.25 X 10^{10} bacteria per ml) for their killed vaccine trial. In this study, 0.3 % of formalin (40%) was used for inactivation. The total volume was 500 ml. The experiment used 1.5 ml of formalin to inactivate the whole culture. This was kept at a temperature of 37^oc for 72 hours to complete inactivation. This is in line with the work of Heddleston and Hall (1958) who used 0.25% of formalin. Arshad (2002) and OIE (2004) recommended use of 0.3 % of formalin for inactivation.

Three dose levels (10^6, 10^9 and 10^{12} CFU/ml) of formalin inactivated locally isolated *P. multocida* vaccines were inoculated in different groups of pullets at the age of 10^{th} week. Antibody response (titer) was measured using IHA test. Reliability of the IHA test for the determination of antibody titer had been advocated by Landy and Trapani (1963); Hussain *et al.* (1979 and 1986); Sawada *et al.* (1985) and Azam *et al.* (1991). This IHA technique is reliable, sensitive, quite accurate and extensively employed for measuring immunity against *P. multocida* (Carter, 1964; Wells *et al*., 1979; Alwis, 1982; Bain *et al.*, 1982; Solano *et al*., 1983). The IHA test and enzyme linked immunosorbent assay were similar in their ability to detect the immune response in the sera as reported by Filion *et al.* (1985).

This study found that the dose level of 10^{12} CFU of *P. multocida* gave significantly higher IHA antibody titers than 10^6 and 10^9 CFU. This helped in deciding the appropriate dose level of bacteria for the preparation of adjuvnted vaccines. This finding agrees with the work of Arshad

(2002) on his study to produce inactivated fowl cholera bactrin. Arshad (2002) found that the dose level of 10^6 CFU elicited least antibody response and dose dependent antibody response was observed as the dose level exceeded from 10^6, 10^9 and 10^{12} CFU. When the dose level exceeded from 10^9 to 10^{12} either no or non-significant decrease in geomean titers was noted.

Formalin inactivated adjuvanted vaccines were inoculated as double dose. Primary and secondary doses were given at 2 weeks interval and revealed significant difference of antibody response after one week of booster vaccination. IHA antibody titers rose significantly at first week of booster dose indicating antigenic potential of the organism. This study was very similar with the works of (Arshed, 2002) who found different IHA responses at different dose levels and adjuvants as well as in primary and booster vaccinations. Arshed (2002) also reported that two doses of vaccine were more effective than one in producing a sustained immunity. However, for the first month of Arshed (2002) study there was no difference in the immune response between primary and booster dose vaccination; but from there onwards to the end of the experiment (25 weeks post-vaccination) the primary dose vaccination rapidly lost effectiveness. Nevertheless, the booster dose vaccination gave satisfactory protection. Nisonaff (1985) and Tizard (2003) described that the secondary immune response is more intense because the initial inoculation of antigen leads to multiplication of responsive cells, which may persist for a long time in the animal. Such investigations of graded dose response correlate well with the work conducted by Hussain *et al.* (1986) and Azam *et al.* (1991).

The Alum precipitated vaccine was found highly irritant and led to somewhat variable results. Hence it is now more usual to add the antigen to preformed Al $(OH)_3$ gel. In this study, the antigen adsorption was standardized at specific PH values and dose of aluminium contents recommended by Bomford (1997) that ranges from 2 to 10 mg per dose of vaccine. Over and above this dose, aluminium contents could reduce the stimulation of immune response. In the present study, the total aluminium hydroxide content per dose of aluminium hydroxide adsorbed vaccine was adjusted to 10 mg. This type of vaccine was a new effort in the immunization of chickens against *P. multocida* in getting 86% protection against challenge. In this trial, the chickens recorded the GMT value of 112 ± 6.5 at 2^{nd} week of post booster that gradually declined. This is in line with the works of Islam (2004). Arshed (2002) found 90-100% protection

to challenge exposure by immunizing chickens against *P. multocida* having aluminium hydroxide adjuvant. In that trial, the chickens that took single dose of aluminium hydroxide adjuvanted vaccine recorded the highest GMT value of 90.5 at 2nd week post vaccination that gradually declined to 18.4 on the 24th week post vaccination. However, the birds vaccinated twice responded with the highest GMT (128.0) on the 4th week post-boostered dose and the lowest GMT value of 73.3 on the 20th week post-booster dose. The birds challenged at this stage (20th week) responded with 90% protection. Matumoto and Yamamoto (1971) reported that $Al(OH)_3$ gel added to the broth bacterin against infectious coryza significantly enhanced its capacity to provoke immunity and exhibited an overall average protection rate of 81.9%. Since, this protective index was based on a quite severe challenge (intra-sinus inoculation of the organism), it might be even more effective under natural conditions of exposure. Heddleston and Reisinger (1960) reported that an aluminium hydroxide adsorbed killed fowl cholera vaccine produced an effective immunity of fowl cholera for at least weeks. Headdleston and Reisinger (1960) were of the view that the aluminium hydroxide adsorbed vaccine did not stimulate a degree of immunity higher than that produced by the water-in-oil emulsified vaccine. However, aluminium hydroxide adsorbed vaccines have the advantages of simplicity of production and ease of administration at low temperatures. The findings in this study indicated that aluminum hydroxide adsorbed vaccine is a lowly effective than oil adjuvanted vaccine. These findings compare well with the results of Bhasin and Biberstein, (1968) who reported that aluminum hydroxide adsorbed bacterins were equal to or even slightly more effective than oil adjuvanted bacterins.

The adjuvant effect of aluminium was manifested primarily in the increase in IgG and was first thought to be due to delayed rate of absorption of the precipitated antigen. An antigen has been shown to persist in aluminium potassium sulphate (alum) precipitated form at the site of injection for at least three weeks (Bunn, 1993). Aluminium potassium sulphate (alum) was the other adjuvant that this trial used. The IHA log titer in the group of birds that took aluminium potassium sulphate adjuvanted vaccine had their peak (GMT=136.0 $\pm$ 7.3) on the 2nd week of post booster vaccination. Thereafter it gradually dropped. During the challenge protection test, the survival of the birds in the group that took aluminium potassium sulphate adjuvanted vaccine was 93.4 % at the 2nd week of post booster vaccination challenge. The birds in this group showed relatively better IHA antibody titers than the birds in trial groups that received booster doses of

fowl cholera vaccines. Aluminium based adjuvants also resulted in getting earlier and better IHA antibody titers.

The Montanide incomplete Seppic adjuvants (ISAs) (Seppic, Paris, France) are a series of adjuvants composed of a variety of oils, different emulsion characteristics, different emulsifiers, and immunomodulators. The Montanide ISA 50 and ISA 70 adjuvants are water-in-oil emulsions based on a purified mineral oil and are similar to FIA (Johnston *et al.,* 1991; Leenaars *et al.*, 1998). The present study utilized montanide ISA 50 as oil adjuvant with 50% proportion to the bacterial culture. Color of oil-emulsified vaccines prepared in this study milky white and emulsion type was water- in- oil. This is exactly the same with the work of (Arshed, 2002) who prepared oil adjuvant fowl cholera vaccine by mixing mineral oil (liquid paraffin) and vegetable oil (coconut oil) as aqueous to oil ratio 1:3. The surfactants, span-80 (oil-phase) and Tween-80 (aqueous phase), were added to reduce the surface activity of oil and aqueous phases and to produce the stable emulsions. It was reported that the vaccines containing only the aqueous phase emulsifier or oil phase emulsifier had much more higher viscosity as compared to those vaccines containing both the emulsifiers (Stone *et al.*, 1978). In the current study, the efficacy of oil adjuvanted vaccine was compared with aluminium salt adjvanted vaccines by inoculating as double dose of these vaccines to various groups of birds and measuring IHA antibody titers and protection against virulent *P. multocida* serotype challenged at 16^{th} week of age (2^{nd} weeks of post booster vaccination). The peak GMT (120 ± 5.3) was at 2^{nd} week of post booster vaccination with gradual drop. On the other hand, 93.4% of birds that took montanide ISA 50 adjuvanted vaccine survived the challenge. These results are in line with the findings of Heddleston and Resisnger (1960) who reported that a bacterin consisting of a water-in-oil emulsion of formalized *P. multocida* was capable of producing a high degree of immunity against fowl cholera in chickens for at least 9 months. It is also in line with the findings of Heddleston *et al.* (1970) and Hofacre *et al*. (1987) who administered two doses of a *P. multocida* bacterin at a two - four week interval and reported as high as 90% and 91% protection respectively. Arshed (2002) reported that birds vaccinated with this vaccine twice showed significantly higher IHA antibody titers (GMT =119.4) and had 100% protection against challenge of *P. multocida* serotype 1. Dua and Maheswaran (1978) reported that the oil adjuvanted bacterin induced statistically significant levels of humoral and cell mediated immune responses as compared to the levels induced by the

live vaccines. In another study Layton (1984) observed a significantly less mortality in immunized ducklings, with a broth-grown *P. multocida* bacteria, than unimmunized controls following virulent challenge.

6. CONCLUSIONS AND RECOMMENDATIONS

Poor husbandry practices, low productive potential of local breed, and various viral and bacterial avian diseases have made the outcome obtained from poultry in Ethiopia below expectation. Fowl cholera is one of the most economically important poultry diseases.

Fowl cholera caused by *P. multocida,* is a severe septicaemic disease of domestic and wild fowl and remains an important havoc for the poultry industry due to high morbidity and mortality. In Ethiopia, even though the frequent complaints by the state and private poultry farms of 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. With this anxiety, this study tried to identify the local pathogenic isolate from natural out-break of fowl cholera and prepared formalin inactivated vaccine with three different bacterial loads and three different adjuvants. Pathogenecity test, vaccine efficacy trial test and challenge protection test were under taken on experimental chickens. The $AlK(SO_4)_2$ adjvanted vaccine at bacterial dose of 10^{12} gave the better IHA titer in comparison with $Al(OH)_3$ and montanide ISA 50 adjuvanted vaccines. In view of these conclusions the following out looks are forwarded:

- There have been economic losses due to the fowl cholera in the intensive and extensive poultry production systems in Ethiopia. But the prevalence of the disease has not yet studied. So the prevalence of the disease should be studied to set appropriate measures.

- Fowl cholera as a disease deserves attention from veterinarians, research and development organizations and from farmers. This is because the epidemiology of the disease in Ethiopia has not been known. As the result, it has been excluded from differential diagnosis as compared with other poultry diseases.

- Although inactivated fowl cholera vaccines have been produced in other countries, the poultry enterprises in Ethiopia continue to experience problems associated with the disease due to unavailability of cost-effective vaccine. To alleviate this problem, the

adoption of this technique used in this trial with eventual production of the vaccine at the National Veterinary Institute is highly recommended.

- This study was planned to identify the strains of *P. multocida* circulating in this country. But due to unavailability of the reference strain, the true strain identification has not been made. Thus, studies must be done to identify the strains. This would help in producing more effective vaccines.

- Because of the short study period, the tests that are helpful in knowing the stability of the vaccine were not done. This test and other remaining tests have to be done in the near future.

- The experience accrued in this trial can be applied in veterinary vaccine production technologies for other bacterial and viral diseases as far as the necessary materials are available.

7. REFERENCE

Abera, M. (2005): Bacteriological study of the respiratory tract of sheep slaughtered at Debre Zeit ELFORA export abattoir, MSc thesis, Addis Ababa University, FVM, DebreZeit.

Akand, M. S. I.; Choudhury, K. A.; Kabir, S. M. L.; Sarkar, S. K. and Amin, K. M. R. (2004): Development of washed cell fowl cholera vaccine in Bangladish, *Int. J. poul. sci.,* **3**: 534-537.

Altman, A. and Dixon, F. J. (1989): Immunomodifiers in vaccines. *Adv. Vet. Sci. Comp. Med.,* **33**: 301-343.

Alwis, M. C. L. D. (1982): Immune status of buffalo caves exposed to natural infection with Haemorrhagic septicemia. *Trop. Anim. Health. Prod*. **14**: 29-30.

Arshed, M. J. (2002): Studies on imunoprophylaxis of fowl cholera, PhD thesis on Veterinary Microbiology, University of Agriculture Faisalabad, Faculty of Veterinary Science, Pakistan.

Ashenafi, H. and Eshetu, Y. (2004). Study on gastrointestinal helminthes of local chickens in central Ethiopia. *Rev. Vet. Med.*, **155**: 504-507.

Azam, M.W.; Hussain, I.; Ashfaque, M.; Siddique, M.; Mahomood, T. and Siddiqui, M.T. (1991): Comparative immunogenicity of sonicated *P. multocida* and formalized bacterin in rabbits. *Pak. Vet. J.,* **11**: 120-122.

Bain, R.V. S.; Alwis, M. C. L. D.; Carter, G. R. and Gupta, B. K. (1982): Haemorrhagic septicaemia. *Anim. Prod. Health.*, **33**: 1-38.

Bhasin, J. L. and Biberstein, E. L. (1968): Fowl cholera in turkeys: The efficacy of adjuvant bacterins. *Avian Dis*., **12**: 159-168.

Bomford, R. (1997): Adjuvants in veterinary vaccines: In vaccine of *P. multocida* and their use in diagnosing fowl cholera in turkeys. *Avian Dis.,* **14**: 24-28.

Bunn, O.T. (1993): Vaccine adjuvants and carriers. In: vaccines for veterinary applications. Butter worth Heinemann Ltd., Oxford. Pp: 295-306.

Carter, G. R. (1964): Correlation between haemagglutinating antibody and mouse protection in anti-*pasteurella* (*P. multocida*) sera. *Can. J. Microbiol.,* **10**: 753-756.

Carter, G. R. (1972): Improved hemagglutination test for identifying type A strains of *P. multocida*. *Appl. Microbiol. Biotechnol.*, **24**, 162–163.

Chap,T. L. (2003): Data transformation: In introductory biostatistics, John Wiley and sons Inc., Hoboken, New Jersey, USA. Pp. 108-370.

Christensen, J. P. and Bisgaard, M. (2000): Fowl cholera. *Rev. Sci. Technol.,* **19**: 626-637.

Chung, J. Y.; Wilkie, I.; Boyce, J. D.; Townsend, K. M.; Frost, A. J.; Ghoddusi, M. and Adler, B. (2001): Role of capsule in the pathogenesis of fowl cholera caused by *P. multocida* serogroup A. *Infect. Immun.*, **69**: 2487-2492.

Collier, L.; Balows, A. and Sussman, M. (1998): Microbiology and microbial infections. 9th ed., Topley and Wilsons, Oxford Univ. Press, New York. Pp: 1203-1207.

Cruickshank, R. (1975): Medical Microbilogy. Vol.11, 12th ed., Churchill Livingston, Edinburgh, London, New York. Pp 456-600.

Crutis, P. E.; Ollehead, G. E. and Ellis, C. E. (1980): Virulence and morphology of *P. multocida* of avian organ. *Vet. Rec*., **107**: 105-108.

CSA (2001): Federal democratic republic of Ethiopia. Central statistical investigatory, Statistical abstract (2001), Ethiopia.

Dalsgaard, K. (1987): Adjuvants. *Vet. Immunol and Immunopathol.,* **17**: 145-152.

Derieux W. T. (1978): Response of young chickens and turkeys to virulent and avirulent *Pasteurella multocida* administered by various route. *Avian Dis.,* **22**: 131–39.

Dividedi, P. N. and Sodhi, S. S. (1998): Morphological, cultural and biochemical characterization of *P. multocida* isolated from poultry. *Poul. Advi.*, **22**: 69-71.

Dua, S. K. and Maheswaran, S. K. (1978): Studies on *P. multocida*. VI: Nature of systemic immunity and analysis of the correlation between levels of immunity induced by various fowl cholera vaccines and protection against challenge. *Avian Dis*., **22**: 748-764.

Fatunmbi, O. O.; Newman, J. A.; Sivanandan, V. and Halvorson, D. A. (1992): Enhancement of antibody response of turkey to trivalent avian influenza vaccine by positively charged liposomal aviridine adjuvant. *Vaccine.*, **10**: 623-626.

Filion. L. G.; Cho, H. J.; Shewen, P. E; Raybould, T. J. G. and Wilie, B. N. (1985): Comparison of serological techniques to measure antibody to *P. haemolytica* AI. *Can. J. Comp. Med*., **49**: 99-103.

Gupta, R. K. and Rost, B. E. (2000): Aluminum compounds as vaccine adjuvants. In:O'Hagan, D. T., Ed. Methods in Molecular Medicine. Vol. 42. Vaccine Adjuvant, Preparation Methods and Research Protocols. Totowa, N.J.: Humana Press. Pp. 65-89.

Hansen, L. M. and Hirsh, D. C. (1989): Serum resistance is correlated with encapsulation of avian strains of *P. multocida*. *Vet. Microbiol*., **21**: 177-184.

Heddleston K. L. (1962): Studies on pasteurellosis. V: Two immunogenic types of *P. multocida* associated with fowl cholera. *Avian Dis.*, **6**: 315–321

Heddleston K. L.; Gallagher J. E. and Rebers P. A. (1972): Fowl cholera: Gel diffusion precipitin test for serotyping *P. multocida* from avian species. *Avian Dis.*, **16**: 925–936.

Heddleston, K. L. and Hall, W. J., (1958): Studies on Pasteurellosis II: Comparative efficiency of killed vaccines against fowl cholera in chickens. *Avian Dis.*, **2**: 322-335.

Heddleston, K. L. and Reisinger, R. C. (1960): Studies on pasterullosis. IV: Killed fowl cholera vaccine absorbed on aluminium hydroxide. *Avian Dis*., **4**: 429-435.

Heddleston, K. L.; Gallagher, J. E and Rebers. P. A. (1970): Fowl cholera: Immune response in turkeys. *Avian Dis*., **14**: 626-635.

Heddleston, K. L.; Watko, L. P. and Reoers, P. A. (1964): Dissociation of a fowl cholera strain of *P. multocida*. *Avian dis*., **8**: 649-657.

Hofacre, C. L. and Glisson, J. R. (1986): A serotypic survey of *P. multocida* isolated from poultry. *Avian Dis*., **30**: 632-633.

Hussain, M.; Cheema, R. A.; Afzal, H.; Akhtar, M. S.; Ijaz, K. and Ahmad, A. (1979): Studies on immunogenicity of the capsular antigen of *P. multocida* in buffalo caves. *J. Anim. Sci*., **1**: 6-10.

Hussain, M. K.; Naeem, W; Pizada, H. and Khan, M. Q. (1986): Immunogenicity of bacterin and capsular antigen of *P. multocida* in buffalo caves. *Pak. Vet. J*., **6**: 51-54.

Islam, M. A.; Samad, M. A. and Rahman, M. B. (2004): Evaluation of alum precipitated formalin killed fowl cholera vaccines with their immunologic responses in ducks. *Int. J. Poul. Sci.*, **3**: 140-143.

Jeurissen, S. H. M.; Janes, E. M.; Ekino, S.; Nieuwenhuis, P.; Kock, G. and Deboer, G. F. (1988): Moncolonal antibodies and probes for defying cellular subscts in bone marrow, thymus, bursa of fabricius and spleen of the chicken. *Vet. Immunol. Immunopathol*., **19**: 225-228.

Johnston, B. A.; Eisen, H. and Fry, D. (1991): An evaluation of several adjuvants emulsion regimens for the production of polyclonal antisera in rabbits. *Lab. Anim. Sci.*, **41**: 15-21.

Landy, M. and Trapani, A. J. (1963): A haemagglutination test for plague antibody with purfied capsular antigen of *pasteurella pestis*. *Am. J. Hyg*., **59**: 150-156.

Layton, H. W. (1984): Efficacy of broth grown *P. multocida* bacterians in ducklings. *Avian Dis.*, **28**: 1086-1095.

Layton, H. W. and Sandhu, T. S. (1984): Protection of duckling with a broth grown *P. antipestifer* bacterin. *Avian Dis*., **28**: 718-726.

Leenaars, P.; Koedam, M.; Wester, P.; Baumans, V.; Claassen, E. and Hendriksen, C. F. (1998): Assessment of side effects induced by injection of differentadjuvant/antigen combinations in rabbits and mice. *Lab. Anim. sci.*, **32**: 387-406.

Lindblad E. B. (2000): Freund's adjuvants. In: O'Hagan, D.T., Ed. Methods in molecular medicine: Vaccine adjuvants, preparation methods and research protocols. Totowa NJ: Humana Press. Pp. 49-63.

Liowa, T. M. and Teo, T. P. (1978): Biochemical studies on *P. multocida* isolated from pigs and poultry. *Sing. Vet. J.*, **2**: 29-32.

Matsumota, M. and Strain, J. G. (1993): Pathogenicity of *P. multocida*, its variable nature demonstrated by in vivo passages. *Avian Dis.*, **37**: 781-785.

Matsumoto, M. and Yamamota, R. (1971): A broth bacterin against infectious coryza. Am. *J. Vet. Res.*, **36**: 579-582.

Merchant, I. A. and Packer, R. A., (1983): Genus Pasteurella. In: Veterinary bacteriology and virology. 7th ed., C.B.S. Publisher and Distributor. Pp. 341-343.

Nisonaff, A. (1985): Introduction to molecular immunology. 2nded., Sinaver Assoc.Inc. Massachusetts, USA. Pp. 50

OIE, (2004): Fowl cholera In: Manual of diagnostic tests and vaccines for terrestrial animals, 5 th ed., World organization of animal health, Paris. http://www.oie.int/eng/normes/mmanual/ A_00112.htm.

OIE. (2000): Principals of veterinary vaccine production. In: Mannual of standards for diagnosis tests and vaccines. World organization of animal health, Paris. Pp.390.

Ozobey, G. and Muz, A. (2006): Isolation of aerobic bacteria from the lungs of chickens showing respiratory disorders and confirmation of *P. multocida* by polymerase chain reaction (PCR). *Vet. arch.*, **76**: 217-225.

Peters, A. R. (1993): Vaccines for veterinary applications. Butterworth Heinemann, Oxford. Pp: 295-306.

Quinn, P. J.; Markey, B. K.; Carter, M. E.; Donnelly, W. J. C. and Leonardo, F. C. (2002): Veterinary microbiology and microbial disease, Blackwell science, London. Pp.1-648.

Radostits, O. M.; Gay, C. C.; Blood, D. C. and Hinchcliff, K. W. (2000): Veterinary medicine. A textbook of the disease of cattle, sheep, pigs, goats, and horses.9th ed., Harcourt publishers limited, London, Philadelphia. Pp.701-867.

Rahman, S.; Ashfaque, M.; Rahman, Z. and Hur, G. (1994): *P. multocida* Robert type-1 antibodies in camels. *Pak. Vet. J*., **14**: 197-199.

Rhodes, K. R. and Rimler, R. B. (1991): Fowl cholera pathogenesis. *Avian dis.*, **34**: 381.

Rimler, R. B. (1994): Presumptive identification of *P. multocida* serogroups A, D, and F by capsule depolymerisation with mucopolysaccharidases. *Vet. Rec.*, **134**: 191–192

Rimler, R. B. and Glisson, J. R. (1997): Fowl cholera. In: Diseases of poultry, 10th ed, Calnek, B. W., Barnes, H. J., Beard, C. W., McDougald, L. R. and Saif, Y. M., Eds. Iowa State University Press, Ames, Iowa, USA. 143–159.

Rimler, R. B. (1987): Cross-protection factors of *P. multocida*: passive immunity of turkeys against fowl cholera caused by different serotype, *Avian Dis*., **31**: 884-887.

Rimler, R. B.; Sandhu, T. S. and Glisson, J. R. (1998): Pasteurellosis, Infectious serositis, and pseudotuberculosis. *In:* A laboratory manual for the isolation and identification of avian pathogens, 4th ed., Iowa state university press, Ames, Iowa, USA. 143–159.

Saville, P. (1995): Fowl cholera, Animal health advisory leaflet 7, South pacific commission, Oceania printers Ltd., Suva, Fiji. http://www.spc.int/rahs/publication/leaflets/AHAL% 2007E.pdf

Sawada, T. R.; Rimler, B. and Rhoades, K. R. (1985): Haemorrhagic septicaemia naturally acquired antibodies against *P. multocida* type B and E in calves in the United States. *Am. J. Vet. Res*., **46**: 1247-1250.

Schmimmel, D. and Van, T. D. (1995): Pasteurellas in the embryonated eggs. I: Virulence checking. *ALTEX*, **12**: 31-33.

Solano,W.; Giambrone, J. J. and Panangola, V. S. (1983): Comparison of ELISA and IHA test for quantitating antibody responses in chicken against *P. multocida*. *Avian. Dis*., **27**: 1034-1042.

Stewart, Tull DES (2003): Adjuvant formulations for experimental vaccines. In: Robinson, A.; Hudson, M. J., Eds. Methods in Molecular Medicine: Vaccine Protocols. 2nd ed. Totowa NJ: Humana Press. Pp.175-193.

Stewart, Tull DES. (2000): Harmful and beneficial activities of immunological adjuvants. In: O'Hagan D. T., Eds. Methods in molecular medicine: Vaccine adjuvants. Preparation methods and research protocols. Totowa NJ: Humana Press. Pp. 29-48.

Stills, H. J. (1994): Polyclonal antibody production. In: Manning, P. J.; Ringler, D. H. and Newcomer, C. E., Eds. The biology of the laboratory rabbit, 2nd ed., San Diego, Academic Press Inc. Pp. 435-448.

Stone, H. D.; Brugh, M.; Hopkins, S. R.; Yoder, H. W. and Beard, C. W. (1978): Preparation of inactivated oil emulsion vaccines with Avian Viral or Mycopasma antigens. *Avian Dis*., **22**: 666-674.

Tadelle, D.; Million, T.; Alemu, Y. and Peters, K. J. (2003): Village chicken production systems in Ethiopia: Use patterns and performance evaluation and chicken products and socio economic functions of chicken. Debre Zeit Agricultural Research Center, Debre ziet, Ethiopia. *Livest. Res. Rural. Dev*. **15.** http://www.cipav.org.co/lrrd/lrrd15/1/tadeb151.htm.

Tadesse, S., Ashenafi, H. and Aschalew, Z. (2005): Seroprevealence study of Newcastle disease in local chickens in central Ethiopia. *Int. J. Appl. Res. Vet. Med*., **3**: 25-29.

Thrusfield, M. (2005): Veterinary Epidemiology. 3rd ed., UK, Blackwell science Ltd, Pp. 233.

Tizard, I. R. (2003): Veterinary immunology: an introduction, 6th ed. W.B. Sounders company, Philadelphia, USA. Pp. 235-252.

Tripathy, D. N.; Hanson, L. E. and Mayrs, W. L. (1970): Passive hemagglutination assay with fowl poxvirus. *Avian Dis.,* **14**: 29-38.

Unaune, E. R. (1984): Antigen presenting function of the macrophages. *Ann. Res. Immunol*., **2**: 395-428.

United States of America Department of Agriculture (USDA) (2001): Code of federal regulations, title 9, animals and animal products. Office of the federal register, National archives and records administration. US government printing office, Washington D.C., USA.

Vanio, O. T.; Vetrola, P.; Toivanen, T. and Ratcliffe, M. J. H. (1988): Antigen presenting cell-T cell interaction in chicken is MHC class II antigen restricted. *Hum. Immunol*. **140**: 2864-2868.

Vanio. O. T. and Ratcliffe, M. J. H. (1984): Proliferation of chicken peripheral blood leukocytes into pokeweed nitrogen is macrophage dependent. *Cell. Immunol*. **85**: 235-243.

Wells, P. W.; Gilmour, N. J. L.; Bureells, C. and Thompson, D. A. (1979): A serological comparison of *P. haemolytica* vaccines containing different adjuvants. *Res. Vet. Sci*., **27**: 247-250.

Whiteman, C. E, and Bickford, A. A. (1989): Avian disease manual, American association of avian pathologists, 3 rd edi., Kendall/Hunt publishing company, Iowa, USA, Pp. 98-102.

Wilson, M. A.; Rimler, R. B. and Hoffman, L. J., (1992): Comparison of DNA fingerprints and somatic serotypes of serogroups B and E *P. multocida* isolates. *J. Clin. Microbiol*. **30**: 1518–1524.

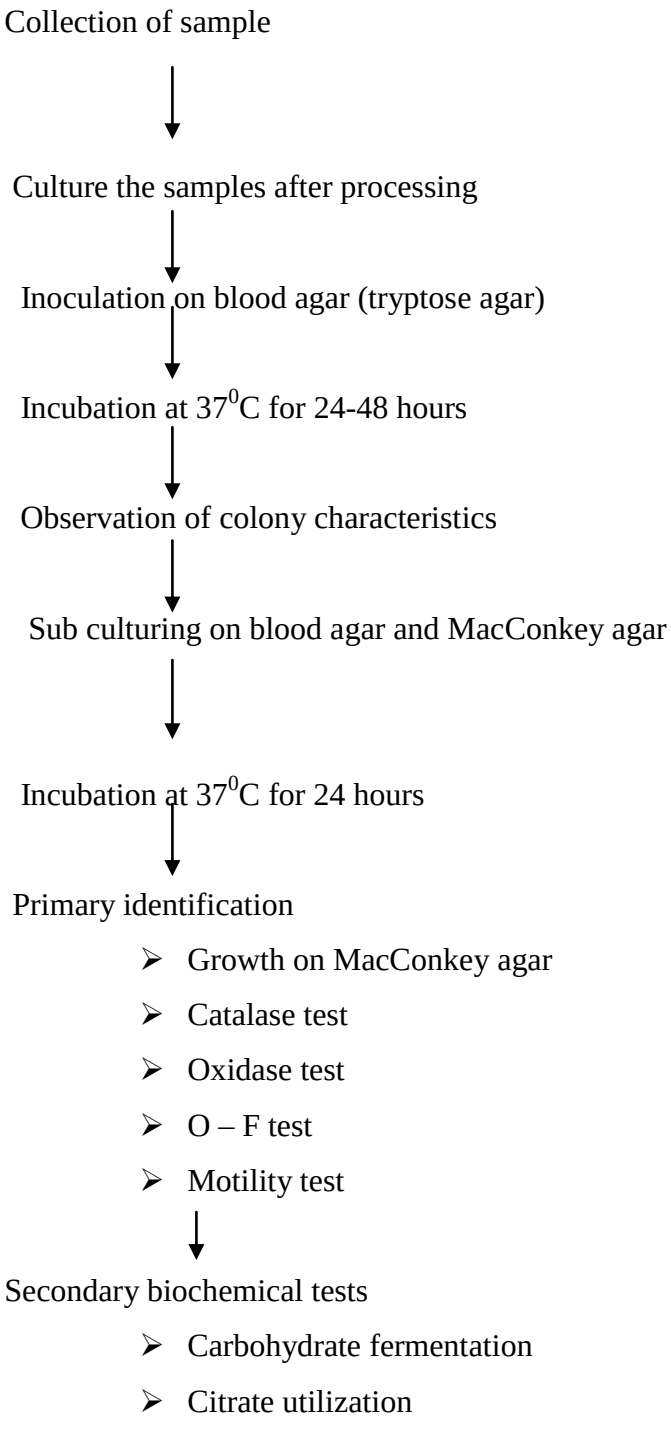
Annex I: Starters feed

Item	Amount
Maize	229 kg
Meat bone	10 kg
Soya been	40 kg
Reap seed	10 kg
Nug cake	85 kg
Wheat bran	30 kg
Wheat middling	35 kg
Molasses	5 litter
Premix 10%	50 kg
Limestone	5 kg
Lycine	100 gram
Choline chloride	100 gram
Methionine	100 gram
Salt	500 gram

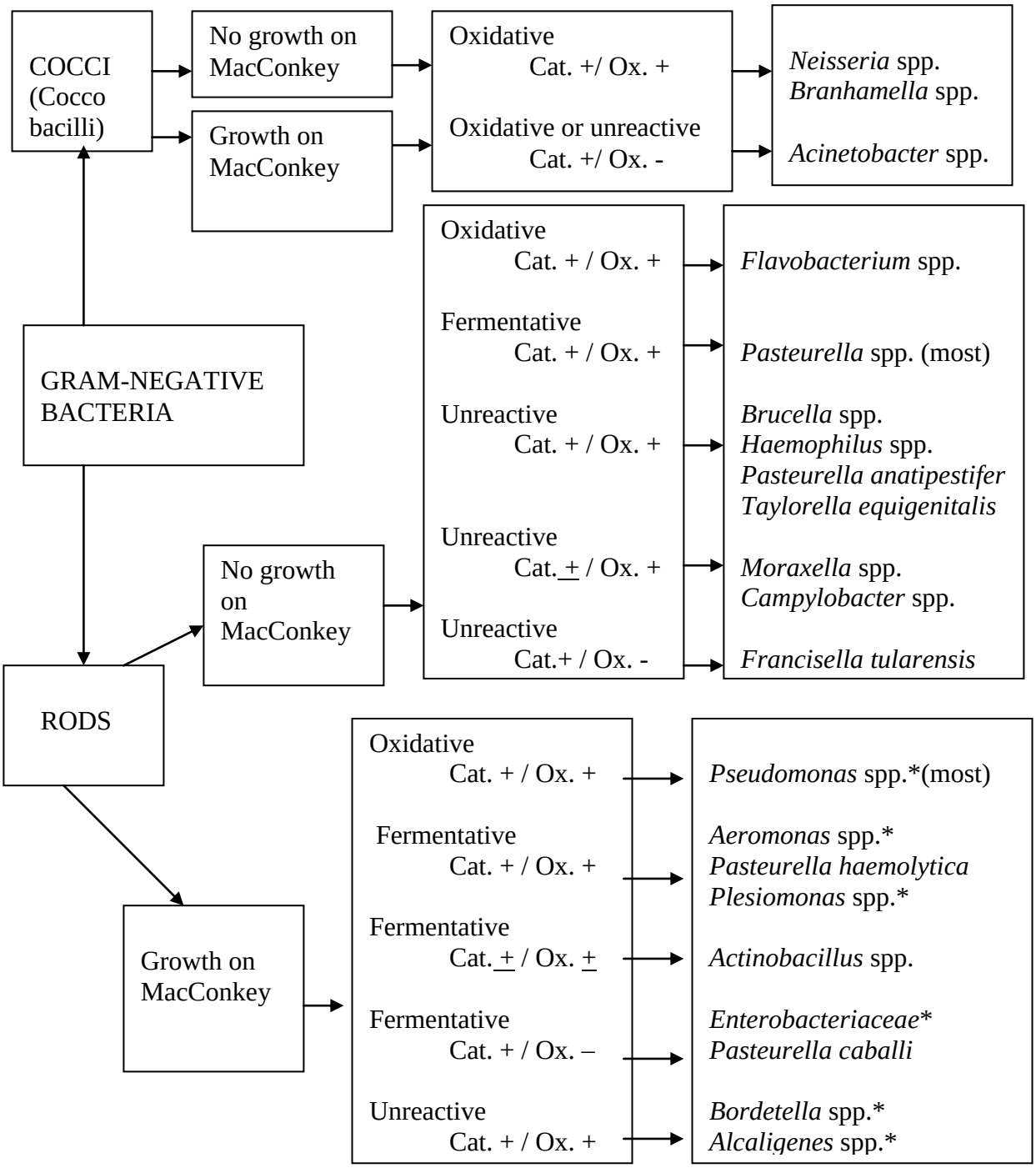
Annex II: Pullets feed

Item	Amount
Maize	244 kg
Meat bone	10 kg
Soya been	35 kg
Reap seed	15 kg
Nug cake	75 kg
Wheat bran	50 kg
Wheat middling	50 kg
Molasses	5 litter
Premix 1%	5 kg
Limestone	10 kg
Lycine	100 gram
Choline chloride	100 gram
Methionine	140 gram
Salt	500 gram

Annex III: Flow chart for isolation and identification of bacteria from poultry samples



Annex IV: Primary identification of Gram-negative bacteria



(Cat. =Catalase; Ox. =oxidase; += positive reaction; - = negative reaction; ± = variable, *= motile

Source: (Quinn *et al*., 2002)

Annex V: Primary identification tests

Gram's stain (Carter, 1984)

Procedure:

- Make a thin smear or film
- Allow the film to dry in air
- Fix the film by passing through the Bunsen flame several times
- Flood the slide with crystal violet for 30 to 60 seconds
- Pour of the stain and wash the remaining stain with iodine solution
- Wash off the iodine and shake the excess water from the slide
- Decolorize with acetone alcohol
- Counter stain with safranin for 30 to 60 seconds and wash with water

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

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

Procedure: A loopful 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% H_2O_2 is added. An effervescence of oxygen gas, within a few seconds, indicates a positive reaction.

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

Principle: The cytochrome oxidase enzyme is able to oxidize the substrate tetramethyl-p-phenylenediamine dihydrochloride,forming a coloured end product, indophenol.

Procedure: Prepare a solution of 1 % tetramethyl-p-phenylenediamine dihydrochloride, then a piece of filter paper is moistened in a Petri dish with fresh reagent and the test bacterium is

streaked firmly across the filter paper with a glass rod. A dark purple colour along the streak line with in 10 seconds indicates a positive reaction. *Pseudomonas aeruginosa* can be used as a positive control organism

O-F test (Quinn *et al*., 2002)

Procedure: Prepare O-F base medium and when the O-F base has cooled to 50 ^{0}C add 20 ml of sterile glucose solution into 200 ml of O-F base, for a final concentration of 1 % glucose and dispense in to tubes. Two tubes of the O-F medium are heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen and the tubes are then cooled rapidly under cold running water. Both tubes are stab-inoculated with the bacterium and a layer of sterile paraffin oil is layered on top of one of the tubes (sealed tube) to a depth of about 1cm and the tubes are incubated at 37^{0}C and examined in 24 hours and then daily for up to 14 days.

Motility test (Quinn *et al*., 2002)

Procedure: SIM medium (BBL) was used to detect motility and the medium was stab inoculated using a straight wire. Then the tube is examined for motility after 24 and 48 hours. If there is a diffuse growth throughout the medium, the bacterium is motile. The growth of a non-motile bacterium is confined to stab line. To interpret the results, hold the tube against a good light and compare the inoculated tubes with un inoculated one.

CAMP test (Quinn *et al*., 2002)

Procedure: A culture of the *Staphylococcus aureus*, with a wide zone of partial haemolysis (beta-haemolysin) is streaked across the center of a sheep or ox blood agar plate. A streak of the suspect Group B streptococcus is made at right angles to, and taken to with in 1 to 1.5 mm of staphylococcal streak.The plate is incubated at 37^{0}C for 18 – 24 hours. An arrow–head of complete haemolysis, indicates a positive CAMP test. The group B streptococci produce diffusible metabolites that complete the lysis of the red cells, only partially haemolysed by the beta –haemolysin of the staphylococcus.

Annex VI: Secondary identification tests.

1. Indole test (Quinn *et al*., 2002)

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

Procedure: Stab inoculate SIM medium with test bacterium and incubate at 37 ^{0}C for 18 to 24 hours. Then add Kovac's reagent (0.2 ml) to tube and stand for 10 minutes.

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

2. Methyl red (MR) test (Quinn *et al.,* 2002)

Principle: It is a quantitative test for acid production, requiring positive organisms to produce strong acids (lactic, acetic, and formic)

Procedure: Inoculate MR-VP broth with pure culture of test organism and incubate at 37^{0}C for two days, then add 5 drops of MR solution into the media.

Interpretation: Production of red colour indicates a positive result and yellow colour negative in methyl red test.

3. Voges-Proskauer (VP) test (Quinn *et al*., 2002)

Principle: Some organisms produce acetoin as the chief end product of glucose metabolism and form less quantity of mixed acids.
Procedure: Inoculate MR-VP broth with pure culture of the test organism and incubate at 37^{0}C for 2 days. Then aliquot 1 ml of broth to a clean test tube and add 0.6 ml of 5 % α-naphthol

followed by 0.2 ml of 40 % KOH. Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 miutes.
Interpretation: A pink colour indicates a positive reaction.

4. Urease test (Quinn *et al*., 2002)

Principle: Urease is an enzyme possessed by many species of microorganism that can hydrolyze urea with the formation of ammonia (alkaline).

Procedure: The surface of the agar slant is streaked with the test organism and incubated at 37^0C for 18 to 24 hours.

Interpretation: Organisms that hydrolyze urea rapidly may produce positive reaction with in 1 or 2 hours. Red (pink) color through out medium indicates positive reaction.

Annex VII: Media used for isolation and identification of bacteria

1. Blood Agar Base (BBL®, Becton Dickinson, USA)

Composition (g/l): Heart muscle, infusion from (solids) 2.0; pancreatic digest of casein 13.0; Yeast extract 5.0; sodium chloride 5.0; agar 15.0

Preparation: Suspend 40.0g of the powder in 1 liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^0C for 15 minutes. Cool the base to 45 to 50^0C and add 5% sterile defibrinated blood.

2. Nutrient agar (Oxoid, Hampshire, England)

Composition (g/l): "Lab-Lemco" powder 1.0; yeast extract 2.0; peptone 5.0; sodium chloride 5.0; agar 15.0.

Preparation: Suspend 28g in 1 liter of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121^0C for 15 minutes.

3. MacConkey Agar (Oxoid, Hampshire, England)

Composition (g/l): Peptone 20.0; lactose 10.0; bile salts No.3 1.5; sodium chloride 5.0; neutral red 0.03; crystal violet 0.001; agar 15.0

Preparation: Suspend 51.5g in 1 liter of distilled water. Bring to boil completely. Sterilize by autoclaving at 121^0C for 15 minutes.

4. Modified Tryptic soy broth with Novobiocine(mTSB+n)

Tryptic soy broth (Oxoid Ltd., Hampshire, England)

Composition (g/liter): Pancreatic digest of casein 15.0, enzymatic digest of soybean meal 5.0, sodium chloride 5.0, Di potassium hydrogen phosphate (K_2HPO_4) 2.5), Glucose (2.5g). Bile salt (Oxoid Ltd., Hampshire, England) 1.5 g, Di potassium hydrogen phosphate (K_2HPO_4) (Techno Pharmaceuticals, Bahadurgarch, India) and Novobiocine (Sigma, Steinheim, Germany)

Preparation: 30 g of the tryptic soy both, 1.5 g of bile salt and 1.5 g of K_2HPO_4 were measured, mixed and dissolved in one liter of distilled water and sterilized by autoclaving at 121^{o} C for 15 minutes. 400 mg of novobiocine in 100 ml of distilled water was mixed, membrane filtered and added to the prepared media tempered at 50 ^{O}C.

5. Tryptic Soy Agar (Difco, Sparks, USA)

Composition (g/liter): Pancreatic digest of casein 15.0; enzymatic digest of soybean meal 5.0; sodium chloride 5.0; agar 15.0

Preparation: forty grams of the powder was suspended in 1 liter of distilled water and mixed thoroughly. The medium was heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Then autoclaved at 121^{o}C for 15minutes, dispensed into cryovial tubes and allowed the medium to solidify.

6. SIM Medium (BBL®, Becton Dickinson, USA)

Composition (g/l): Pancreatic digest of casein 20.0; peptic digest of animal tissue 6.1; ferrous ammonium sulfate 0.2; agar 3.5

Preparation: Suspend 30g of the powder in 1 liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for 1minute. Autoclave at 121^{0}C for 15 minutes.

7. O-F basal medium (Merck, Darmstadt, Germany)

Composition (g/l): Peptone from casein 2.0; yeast extract 1.0; sodium chloride 5.0; Di-potassium hydrogen phosphate 0.2; bromothymol blue 0.08; agar-agar 2.5.

Preparation: Suspend 11g in 1 liter of distilled water by heating in a boiling water bath or in a current steam, autoclave (15 minutes at 121^0C); at approximately 50^0C mix in 100ml/ liter of filter sterilized 10% solution of D (+) glucose, lactose, sucrose, or other carbohydrates; dispense into tubes to give a depth of approximately 5cm.

8. Phenol-red broth base (Merck, Darmstadt, Germany)

Composition (g/l): Peptone from casein 5.0; peptone from meat 5.0; sodium chloride 5.0; phenol red 0.018.

Preparation: Suspend 15g in 1liter of distilled water; dispense into tubes and insert fermentation tubes; if necessary; autoclave (15 minutes at 121^0C). At less than 60^0C add the reactants (final concentration 5 to 10g / liter) as sterile solutions.

9. Triple sugar iron agar (Merck, Germany)

Composition (g/l): Peptone from casein 15.0; peptone from meat 5.0; meat extract 3.0; sodium chloride 5.0;lactose 10.0; sucrose 10.0; D (+) glucose 1.0; ammonium iron (III) citrate 0.5; sodium thiosulfate0.5; phenol red 0, 0024; agar –agar 12.0.

Preparation: Suspend 65g in 1 liter of distilled water by heating in a boiling water bath or in a current steam; dispense into tubes; autoclave (15 minutes at 121^0C). Allow to solidify and give agar slants.

10. Simmons Citrate Agar (BBL®, Becton Dickinson, USA)

Composition (g/l): Ammonium dihydrogen phosphate 1.0; dipotassium phosphate 1.0; sodium chloride 5.0; sodium citrate 2.0; magnesium sulfate 0.2; agar 15.0; bromothymol blue 0.08.

Preparation: Suspend 24.2g of the powder in 1 liter of distilled water. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121^0C for 15 minutes. Cool tubed medium in a slanted position for slants.

11. MR-VP medium (Oxoid, Hampshire, England)

Composition (g/l): Peptone 7.0; Glucose 5.0; Phosphate buffer 5.0

Preparation: Suspend 17g in 1 liter of distilled water. Mix well, distribute into final containers and sterilize by autoclaving at 121 ^{0}C for 15 minutes.

12. Urea Agar Base (BBL®, Becton Dickinson, USA)

Composition (g/l): Pancreatic Digest of Gelatin 1.0; Dextrose 1.0; Sodium Chloride 5.0; Potassium Phosphate 2.0; Urea 20; Phenol red 0.012.

Preparation: Suspend 29g of the powder in 100 ml of distilled water. Mix thoroughly and sterilize by filtration. Suspend 15g of Agar in 900 ml distilled water. Autoclave at 121^{0}C for 15 minutes. Cool to 50^{0}C and add 100 ml of urea agar base. Mix thoroughly and dispense aseptically in sterile tubes. Cool tubed medium in a slanted position so that deep butts are formed.

13. Vian-de foie (VF) media

No	Chemical	Unit	Quantity	Remark
1	Glucose	Gram	5	
2	L-cystine	Gram	0.5	
3	Meat extract broth	Litter	1	
4	Paraffin oil	ml	1	
5	Yeast extract	Gram	3	
6	Minced meat and Liver cooked	4 gram 1 gram		A layer of 4mm/ test tube

Sterilization is by autoclaving at a temperature of 115^{o}c for 30 minutes

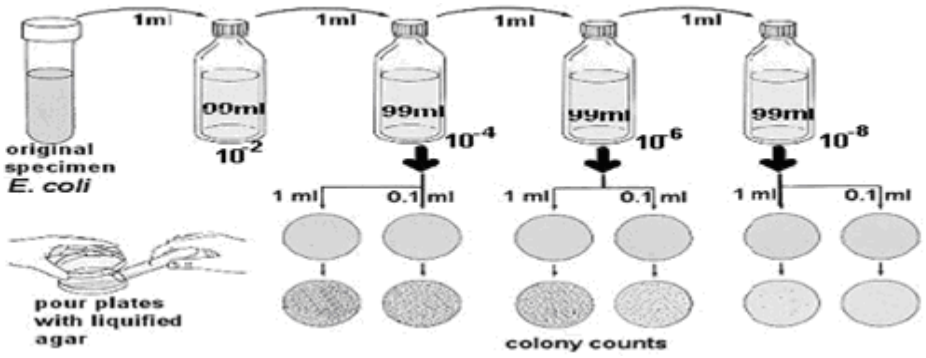
14. Phosphate Buffer Saline With Tween 20(Sigma chemicals Co. Saint Louis, USA)

Composition (0.01MPBS / L): Sodium chloride, 0.138M; Potassium 0.0027M; Tween 200.05%.

Preparation: one sachate of this composition was suspended in 1 liter of distilled water and mixed thoroughly. Sterilize by autoclaving at 121^{o}C for 15 minute. Gently shacked and poured to sterile universal tubes near a flame.

Annex VIII: Standard plate count method of bacterial counting

- Label the bottom of six Petri plates 1-6.Label four tubes of saline 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}., 10^{-10} and 10^{-12}
- Using aseptic technique, the initial dilution is made by transferring 1 ml of *P. multocida* sample to a 99ml tryptose broth (figure below. This is a 1/100 or 10^{-2} dilution.
- The 10^{-2} dilution is then shaken by grasping the tube between the palms of both hands and rotating quickly to create a vortex. This serves to distribute the bacteria and breakup any clumps.
- Immediately after the 10^{-2} dilutions have been shaken, uncap it and aseptically transfer 1ml to a second 99ml tryptose broth. Since this is a 10^{-2} dilution, this second blank represents a 10^{-4} dilution of the original sample.



- Shake the 10^{-4}dilution vigorously and transfer 1ml to the third 99ml tryptose. This third dilution represents a 10^{-6}dilution of the original sample. Repeat the process once more to produce a 10^{-8}dilution continue until 10^{-12}.
- Shake the 10^{-4}dilution again and aseptically transfer 1.0 ml to one petri plate and 0.1ml to another Petri plate. Do the same for the $10^{-6,}$ 10^{-8} ,10^{-10} and the 10^{-12} dilutions.
- Remove one agar pour tube from the 48 to 50 ^{0}C water bath. Carefully remove the cover from the 10^{-4} Petri plate and aseptically pour the agar into it. The agar and sample are immediately mixed gently moving the plate in a figure-eight motion or a circular motion while it rests on the tabletop. Repeat this process for the remaining plates.
- After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 25^{0}C for 48 hours or 37^{0}C for 24 hours.

- ❖ At the end of the incubation period, select all of the Petri plates containing between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC). Plates with fewer than 30 colonies are designated too few to count (TFTC). Count the colonies on each plate. A Quebec colony counter should be used.
- ❖ Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar.

$$\text{No of bacteria/ml} = \frac{\text{No of colonies (CFUs)}}{\text{Dilution X amount plated}}$$

- ❖ Record your results.

Source: Queen *et al*., (2004).

Annex IX: Utilization of freez drier sogev

1. Open the water tap
2. The electric current must be on (red)
3. Close the vacume valve on the back of the freez drier
4. Lift up the capsulating system of apparatus
5. Put the the interrupter on position “congelation”wait 1 hour the temperature reads $35^{o}c$
6. Put the biological to be freeze dried and leave it for 3 hours.
7. Put the interrupter on position “Fd-piege”
8. Wait for ½ hour and the temperature reads 45 ^{o}c.
9. Put the position of the interrupter on “ P vide”
10. Wait 24-48 hours till the reading on TH-chauffage comes around 20 ^{o}c.
11. If the temperature doesnot reach 20 ^{o}c even after 48 hours then put the interrupter on position “chauf ”
12. Leave it for 1 hour.
13. Close the stoppers.
14. Out the vacuum
15. Stop the apparatus
16. Close the water
17. Cut the current flow

Annex X: Serum agglutination test

The principle of the agglutination test is based on the presence of corpuscular antigen (such as bacteria), which is complex, by specific antibodies forming an antigen antibody – network. This results in visible clumping of the antigen. By gravity these clumps are deposited on the bottom of the microtiter cup clearing the formerly turbid supernatant. The SAT was formerly performed in glass tubes. This recipe has adapted the procedure to microtiter dimensions and equipment. A brucella system is used as example.

Equipments

Incubator
Adjustable micropipettes 10-1 00 1;
Multichannel pipettes, adjustable, preferably 10-50 *ul* of both 8 and 12 channels.
Microtiter plates, normally with U-shaped cups (U-type), else wise V-type may be used.

Buffers and reagents, Normal Saline

NaCl	9.0 gram
Distilled water ad.	1000 ml

P. multocida antigen positive and negative control sera

Antigen titration

Add 50 *ul* normal saline is placed into every cup of a microtiter plate. 50 ul of positive control serum at an estimated starting dilution 1:2.5 or 1:5 is placed into all cups of column 1. After mixing the control serum is serially diluted by 8-chanel multichanel pipette in 50ul amounts from column 1 to Column 12 from where 50 ul are discarded. Antigen to be tested is serially diluted in 8 tubes or cups of another microtiter plate from an estimated starting dilution 1:2.5 or 1:5. 50 ul of antigen per cup is added, starting with the last tube/cup of the antigen dilution for row H 1-12, for the last tube/cup for row G1-12 and so on. The amount of antigen per cup is again 50ul. Mix

well and incubate at 37°C over night. The final Volume of the reactant mixture *is* 100 ul, and by the addition of Second reagent all di1utions are doubled.

Test procedure

1. Addition of diluents 80ul of normal saline to all wells of column I; and 50ul to others
2. Addition of control sera (+/-) 20 ul to Al, B1, to H1 and serum samples.
3. Serial dilution of control sera and serum samples, transfer 50ul from Column 1-2,3-12, discard 50ul from 12.
4. Addition of Ag at working dilution 50ul of the antigen diluted in normal saline to all wells and incubate covered plate overnight at 37°C.
5. Reading of the results:

+ Positive is stationary agglutinate
- Negative moves when plate is held upright.

Annex XI: Multiple comparison of the log titer with days in week.

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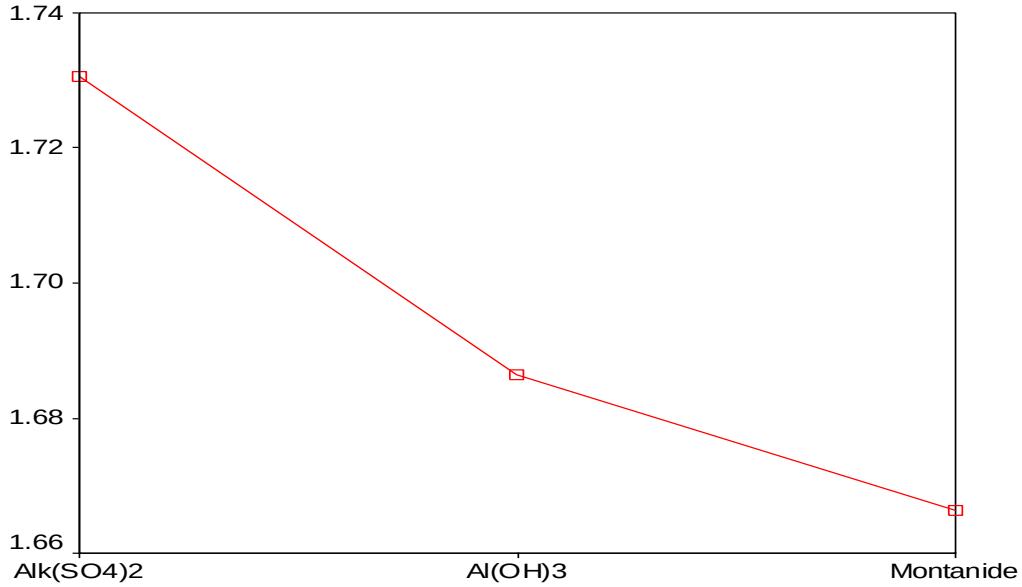
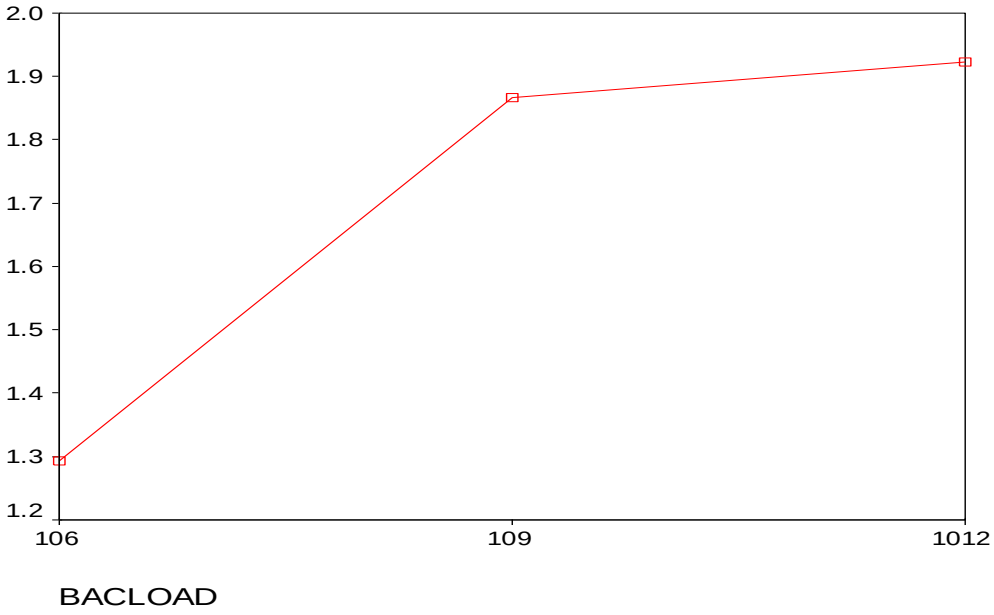
(I) DAY	(J) DAY	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1week	2 week	-.17(*)	.050	.001	-.27	-.08
	3week	-.15(*)	.050	.003	-.25	-.05
	4 week	-.33(*)	.050	.000	-.43	-.24
	6 week	-.27(*)	.050	.000	-.37	-.17
2 week	1week	.17(*)	.050	.001	.08	.27
	3week	.02	.050	.640	-.07	.12
	4 week	-.16(*)	.050	.001	-.26	-.06
	6 week	-.10	.050	.053	-.20	.00
3week	1week	.15(*)	.050	.003	.05	.25
	2 week	-.02	.050	.640	-.12	.07
	4 week	-.18(*)	.050	.000	-.28	-.09
	6 week	-.12(*)	.050	.016	-.22	-.02
4 week	1week	.33(*)	.050	.000	.24	.43
	2 week	.16(*)	.050	.001	.06	.26
	3week	.18(*)	.050	.000	.09	.28
	6 week	.06	.050	.205	-.03	.16
6 week	1week	.27(*)	.050	.000	.17	.37
	2 week	.10	.050	.053	.00	.20
	3week	.12(*)	.050	.016	.02	.22
	4 week	-.06	.050	.205	-.16	.03

* The mean difference is significant at the .05 level.

Annex XII: Line plots that show IHA log titer at different kinds of bacteria load and adjuvant





CURRICULUM VITAE

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- Undergraduate Seminar Paper: The current status of embryo transfer technology
- DVM Thesis: The study on reproductive health problems in indigenous and cross breed cattle in and around Bedelle, west Ethiopia.

- Postgraduate Seminar Paper: contagious bovine pleuropneumonia, the epidemiology and the Ethiopian strategy to the survey and control.
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8. SIGNED STATEMENT OF DECLARATION

I under sign, declare that this thesis is my original work, has not been presented for the degree in any other university.

Name: Molalegne Bitew Aserese

Signature----------

Date of submission----------

This thesis has been submitted for examination with our approval as university advisors.

Academic Advisors	Signature
Mosses Kyule (BVM, MSc, MVPM, PhD)	----------
Kelay Belihu (DVM, PhD)	----------
Berhie Gebre-Egziabher (DVM, MSc, PhD)	----------