

**ADDIS ABABA UNIVERSITY**

**COLLEGE OF HEALTH SCIENCE**

**SCHOOL OF VETERINARY MEDICINE**

**BY**

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**BACTERIOLOGICAL AND SEROLOGICAL INVESTIGATION OF FOWL CHOLERA  
IN SELECTED COMMERCIAL POULTRY FARMS IN DEBRE ZEIT**

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**DEREJE BAYE WUBIE**

**JUNE, 2010**

**DEBRE ZEIT, ETHIOPIA**

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**DEBRE ZEIT, ETHIOPIA**

# BACTERIOLOGICAL AND SEROLOGICAL INVESTIGATION OF FOWL CHOLEA

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Much of my heart felt thanks goes to the Amhara Regional State Bureau of Agricultural and Rural Development and Andassa Poultry Multiplication Center, for giving this scholar ship to carry on my post graduate study on Tropical Veterinary Microbiology.

I extend especial thanks to Genesis and Alema farms for their kind cooperation through out study period.

I am extremely thankful to W/ro Selam Tariku, from Microbiology Laboratory of National Veterinary Institute (NVI), W/ro Almaz Sharew and Ato Kinfemichael Mekuria from Immunology and Virology Laboratory of NVI for their cooperation in performing serological test.

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## LIST OF ABBREVIATIONS

|          |                                            |
|----------|--------------------------------------------|
| AGID     | Agar gel immune diffusion                  |
| bp       | Base pair                                  |
| CI       | Confidence interval                        |
| CSA      | Central statistics authority               |
| CU       | Clemson University                         |
| DNA      | Deoxyribonucleic acid                      |
| DVM      | Doctor of veterinary medicine              |
| FVM      | Faculty of veterinary medicine             |
| ELISA    | Enzyme linked immunosorbent assay          |
| I –ELISA | Indirect enzyme linked immunosorbent assay |
| IHA      | Indirect haemagglutination                 |
| LPS      | Lipo polysaccharide                        |
| MS       | Microsoft                                  |
| NVI      | National veterinary institute              |
| °C       | Degree centigrade                          |
| OF       | Oxidation fermentation                     |
| OIE      | Office international des epizooties        |
| OR       | Odds ratio                                 |
| PBS      | Phosphate buffer saline                    |

|      |                                        |
|------|----------------------------------------|
| PBSS | Phosphate buffer saline solution       |
| PCR  | Polymerase chain reaction              |
| PCV  | Packed cell volume                     |
| PHT  | Passive haemagglutination test         |
| RBC  | Red blood cells                        |
| RPM  | Revolution per minute                  |
| SIM  | Sulphide, Indole, Motility             |
| Spp. | Species                                |
| SPSS | Statistical Package for Social Science |
| TSA  | Tryptic soya agar                      |
| TSI  | Triple sugar iron                      |
| UK   | United Kingdom                         |
| μl   | Micro liter                            |

## ABSTRACT

Bacteriological and serological investigation of fowl cholera was conducted in two commercial poultry farms in Debre Zeit. Cross sectional with continuous follow up of the farms was used to determine the prevalence rate of *P. multocida* isolates from nasal swabs of apparently healthy fowls (384), nasal and tracheal swabs of fowls diseased with respiratory disease syndrome (35), pneumonic lung of fowls died of respiratory syndrome (30) and pneumonic lungs from fowls with typical clinical signs of chronic fowl cholera (5). Cross sectional study was also used to determine seroprevalence of *P. multocida* serotype A from fowls' sera (384). A total of 82 (17.16%) isolates of *P. multocida* were detected from nasal swabs of apparently healthy, fowls diseased with respiratory disease syndrome, tracheal swabs and pneumonic lungs of fowls died of respiratory disease syndrome and from pneumonic lungs of tentatively diagnosed cases of chronic fowl cholera. *P. multocida* was isolated at a rate of 14.58% and 25.75% from nasal swabs of healthy and diseased fowls, respectively, with a statistically significant difference ( $P < 0.05$ ). All isolates were identified upto subspecies level. *P. multocida* subspecies *gallicida* was identified at a rate of 69.64% and 88.46%, while *P. multocida* subspecies *septica* was 30.36% and 11.54% prevalent in apparently healthy and diseased fowls, respectively. The isolation rate of *P. multocida* from pneumonic lungs and their corresponding tracheal swabs showed higher in layers 37.5% and 18.75%, while in growers the rates were 14.2% and 7.14%. There was statistically significant difference ( $P < 0.001$ ) of the isolation rate of the organism between pneumonic lungs and their corresponding tracheal swabs of the two different age groups. A total of 384 sera were also examined by indirect haemagglutination test (IHA) to determine the seroprevalence of fowl cholera in the study farms. Variation in seroprevalence among farms and age groups was observed ( $P < 0.01$ ). The IHA test revealed that higher seroprevalence was observed in farm 2 and in layers with seroprevalence of 28.78% and 26.92%, respectively. While low seroprevalence was observed in farm 1 and in growers with seroprevalence of 8.60% and 9.65%, respectively. Strength of association between risk factors in seroprevalence was computed by using univariable logistic regression and the odds ratio (OR) showed that layers were 3.446 times at risk of fowl cholera than growers, while farm 2 was 3.18 times at risk of fowl cholera than farm 1.

**Keyword:** Bacteriological diagnosis, fowl cholera, *P. multocida*, pneumonic cases, prevalence, serological test, swabs

Chicken occupies a very special part of our economy for being affordable, easily manageable and fast growing compared with other species of animals that provide protein. It is among the most popular meat and egg sources in the world, particularly in developing countries (Sotgiu, 1997). The total poultry population of Ethiopia is estimated at 10.5 million, which represents 60% of the total livestock population in East Africa. Hailu (1992) reported that about 80% of the poultry population in Africa is kept in indigenous management systems. For example, from the total population of chickens in Ethiopia, 90% are raised under the traditional backyard system of management, while 10% are under intensive management system (Tilahun et al., 2003; Ashenafi, 2000; Ashenafi, 2003).

In village systems, farmers keep poultry for diverse objectives. They are raised for purposes of hatching, sale and home consumption, sometimes for feeding construction and gift (Aksew, 1993). Village chicken products are often the only source of animal protein for resource poor households. Eggs are source of high quality protein for sick and malnourished children (Kilalo, 1997; Hailu, 1992).

In Ethiopia, backyard chickens have been raised for long time for similar purposes. They have their own contribution to the country's economy. This is not because they are productive but are huge in number (Ashenafi, 2003; Kilalo, 1997). It is quite evident that poultry farms are flourishing today but in the past mostly extensive type of production was predominant because the major part of poultry production was occupied by individual farmers and consequently, the outcome as a whole was below expectations and limited. Constraints which restrict the potential of backyard chickens in Ethiopia include the presence of diseases of various nature, poor management lack of appropriate selection and breeding and low level of feeding (Ashenafi, 2003; Ashenafi, 2000).

In recent years, the Ethiopian government has initiated a number of activities to improve the productivity and health of the poultry sector. Various measures have been taken to enhance the productivity and health of the sector. One of the major activities undertaken in the sector is the establishment of the National Poultry Research and Training Center (NPRTC) in Addis Ababa. The NPRTC is a government-owned institution that is dedicated to the improvement of the poultry sector in Ethiopia. It has a number of research and training programs that are aimed at improving the productivity and health of the sector. One of the major activities undertaken by the NPRTC is the establishment of the National Poultry Reference Laboratory (NPRL). The NPRL is a government-owned laboratory that is dedicated to the diagnosis and control of poultry diseases. It has a number of diagnostic services that are aimed at improving the health of the sector. One of the major activities undertaken by the NPRL is the establishment of the National Poultry Reference Laboratory (NPRL). The NPRL is a government-owned laboratory that is dedicated to the diagnosis and control of poultry diseases. It has a number of diagnostic services that are aimed at improving the health of the sector.

# 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. It plays major role in the economy particularly of developing countries (Sonaiya, 1990). The total poultry population of Ethiopia is estimated at 56.5 million, which represents 60% of the total chicken population in East Africa. Horst (1989) reported that about 80% of the poultry population in Africa is kept in indigenous production system, for example, from the total population of chicken in Ethiopia, 99% are raised under the traditional back yard system of management, while 1% is under intensive management system (Tadelle *et al.*, 2003; Ashenafi and Eshetu, 2004).

In village systems, farmers keep poultry for diverse objectives. They are raised for purposes of hatching, sale and home consumption, sacrifices for healing ceremonies and gift (Alemu, 1995). Village chicken products are often the only source of animal protein for resource poor households. Eggs are source of high quality protein for sick and malnourished children (Kitalyi, 1997; Horst, 1989).

In Ethiopia, backyard chickens have been reared for long time for similar purposes. They have their own contribution to the country's economy. This is not because they are productive but are huge in number (Alamargot, 1987; Kitalyi, 1997). 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. Constraints which restrict the potential of backyard chickens in Ethiopia include the presence of diseases of various natures, poor management, lack of appropriate selection and breeding, and low input of feeding (Alemu, 1995; Ashenafi, 2000).

In recent years, however, attempts are underway to enhance the poultry productivity and optimize the contribution of chickens to the national economy. Greater efforts have been made to transform the production system into a more commercialized and intensive large scale system (Tadelle *et al.*, 2003; Ashenafi and Eshetu, 2004). In addition, exotic breeds and cross breeds are

being multiplied in government and private owned poultry farms and distributed to individual farmers via the extension division of Ministry of Agricultural and Rural development to be maintained and produced under the backyard management system (Ashenafi, 2000). This is aimed to improve the livelihood and nutrition of poor farmers and further to contribute to the national economy at large (Dessie and Ogle, 2001).

It is a growing concern, however, that there is expansion of diseases of various etiologies into several poultry farms. Furthermore, intensification is aggravating the rapid spread of many infectious diseases between and within poultry farms (Alamargot, 1987; Zeleke *et al.*, 2005). Among 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 in Ethiopia. (Tadesse *et al.*, 2005).

Fowl cholera, caused by *Pasteurella multocida*, is a severe septicemic disease of domestic and wild fowls 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, fowl cholera still remains a problem in the modern poultry industry in developed and developing countries (Merchant and Packer, 1983).

In Ethiopia, fowl cholera causes high economic loss in state and private poultry farms due to high morbidity, mortality, loss of production and high treatment cost, but the prevalence of the disease has not been quantified.

So the present study was planned with the following main objectives:

- To isolate and characterize *P. multocida* from nasopharyngeal swabs of apparently healthy fowls, tentatively diagnosed cases of fowl cholera and from fowls died of pneumonic cases.
- To determine seroprevalence of the disease in the study area.

## 2. LITERATURE REVIEW

### 2.1. Fowl cholera

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 musculo skeletal system are often the seats of chronic infection (Radostits *et al.*, 2000). Common synonyms for fowl cholera are avian pasteurellosis and avian hemorrhagic septicemia.

Fowl cholera is not considered to have zoonotic potential, as avian isolates are generally non pathogenic in human beings 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 MacConkey agar. It is a facultative anaerobic bacterium that grows best at 35 – 37°C. Primary isolation is usually accomplished by using media such as blood agar, tryptic soya agar, and tryptose soya broth. In tryptose soya broth, a uniform turbidity developed and changed the color of the medium from light yellow to dirty white. On tryptic soya agar, convex 2 - 3mm diameter was observed. 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 3mm in diameter after 18 - 24 hours of incubation. They are usually discrete, circular, convex, translucent, and butyrous. Capsulated organisms usually produce larger colonies than those of non 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 - 2.5µl in size. Stained gram negative, and generally occur singly or in pairs. Recently isolated organisms or those found

in tissue smears show bipolar staining with Wright, Geimsa stains or methylene blue, and are usually encapsulated. 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 (Radostits *et al.*, 2000).

Capsular serogrouping and somatic serotyping accomplish antigenic characterization of *P. multocida*. Capsular serogroups are determined by passive haemagglutination test (PHT) (Carter, 1972). Sero groups A, B, D, E, and F have been reported, all but serogroup E have been isolated from avian hosts. None serological disk diffusion test that uses specific mucopolysaccharides to differentiate serogroups A, D, and F have been developed (Rimler, 1994). Somatic serotypes are usually determined by an agar gel immuno diffussion (AGID) test (Heddlestone, 1962; Heddlestone *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 serogroups and serotypes. 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 resulting in bio or trinomial serotypes, as illustrated by the 3, 4 and 3,4,12 serotypes (Rimler *et al.*, 1998).

Identification is based primarily on the result of biochemical tests. Carbohydrate fermentation reactions are essential (Quinn *et al.*, 2002). *P. multocida* ferments glucose, mannose, galactose, fructose, and sucrose. It does not ferment rhamnose, cellobiose, raffinose, inulin, erythritol, adonitol, 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. It produces catalase, oxidase, and ornithine decarboxylase, but not urease, lysine decarboxylase, beta-galactosidase or arginine decarboxylase. Phosphatase production is variable. Nitrate is reduced; indole is produced, but not H<sub>2</sub>S.

#### 2.1.2. Occurrence

Fowl cholera is a disease of many kinds of birds, e.g., chickens, turkeys, geese, ducks, canaries and many wild and zoologic birds. Perhaps all birds are susceptible under appropriate conditions.

In poultry, most outbreaks 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 water fowls and often causes extensive lose among wild water fowls. Geese are highly susceptible. Fowl cholera is more likely to occur in birds that are stressed by such factors as poor sanitation, parasitism, malnutrition and other diseases (Radostits *et al.*, 2000). The occurrence of fowl cholera is world wide and a relatively common disease.

#### 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. Epidemiology

Recovered or sub clinically infected birds harbor the organism in the upper respiratory tract excretions, especially those from the nostrils, mouth and eyes, contaminated feed and water exposing susceptible birds to infection for new birds. Wild birds, including sparrows and pigeons, and many mammals, especially pigs, cats, 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 humeral immunity. Immuno depression increase susceptibility. *P. multocida* is resistant enough to be readily spread on contaminated creates, feed bags, shoes, equipment (Whiteman and Bickford, 1989).

#### 2.1.5. Pathogenesis

*Pasteurella* plays a major role as a secondary pathogen in the final progression of severe pneumonic and septicemic diseases in fowls (Rhodes and Rimler, 1991). Their pathogenesis involves many predisposing agents such as viruses, other bacterial diseases, parasites, environment (excessive temperatures, sudden changes of feed, dust) or stress associated during transportation, mixing of different breeds and ages (Yates *et al.*, 1983; Dung worth, 1992; Lopez, 2001). These factors seem to alter the upper respiratory tract epithelium allowing *P. multocida* to colonize, escaping clearance, and to move from the nasopharynx to the lungs, leading to pneumonia which is accompanied by high morbidity and mortality (Vogel and Parrot, 1994; Edwards, 1996).

#### 2.1.6. Clinical symptoms

Acute fowl cholera often present for only a few hours before death. Unless infected birds are observed during this period, death may be the first indication of disease. Signs that often occur are fever, anorexia, ruffled feathers, mucous discharge from the beak, diarrhea and increased respiratory rate. Cyanosis often occurs immediately prior to death and is most evident in unfeathered areas of the head, such as comb and wattles. Fecal material associated with the diarrhea is initially watery and whitish in color but later becomes greenish and contain mucus (Barnes *et al.*, 1998).

Chronic fowl cholera may follow an acute stage of the disease or result from infection with organisms of low virulence. Signs are generally related to localized infections (Calnek *et al.*, 2001). Wattles, sinuses, legs or wings, joints, foot pads, and sternal bursae often become swollen. Exudative conjunctival and pharyngeal lesions may be observed, and toriticollis sometimes occur (Barnes *et al.*, 1998).

#### 2.1.7. Gross lesions

Lesions of fowl cholera are not constant but vary in type and severity. The greatest variation is related to the course of the disease, whether acute or chronic (Barnes *et al.*, 1998). In acute cases, most of the postmortem lesions are associated with vascular disturbances. Generally, hyperemia

usually occurs, most evident in veins of the abdominal viscera, and may be quite pronounced in small vessels of the duodenal mucosa. Petechial and ecchymotic hemorrhages are frequently found and may be widely distributed. Subepicardial and subserosal hemorrhages are common, as are hemorrhages in the lung, abdominal fat and intestinal mucosa. Increase amounts of pericardial and peritoneal fluid frequently occur. Liver of acutely affected birds may be swollen and usually contain multiple small focal areas of coagulative necrosis. Ovaries of laying hens are commonly affected, mature follicles often appear flaccid. Yolk material from ruptured follicles may be found in the peritoneal cavity. Immature follicles and ovarian stroma is often hyperemic (Randall, 1991).

Chronic fowl cholera is usually characterized by localized infections, in contrast to the septicemic nature of the acute disease. They often occur in the respiratory tract and may involve any part, including sinuses and pneumatic bones. Infections of conjunctive and adjacent tissues occur and facial edema may be observed. Localized infections may also involve the hock joints, sternal bursa, foot pads, peritoneal cavity, and oviduct (Randall, 1991).

## **2.2. Diagnosis**

### **2.2.1. Field diagnosis**

Tentative field diagnosis can be made on the bases of the history, clinical signs, post mortem gross pathological findings and epidemiological features, including species, age groups, season associated with management factors (OIE, 2004). Clinical symptoms including dyspnea, swelling of the comb and wattle, oculonasal discharge and fever ranging from 42.5 - 44<sup>0</sup>C are the most prominent symptoms (Gilmour *et al.*, 1993).

### **2.2.2. Bacteriological diagnosis**

In pneumonic fowl cholera the organism is present in large numbers in lung lesions and exudates. In the acute and generalized (septicemia) form, it can be recovered from the liver, spleen, kidney could be submitted for isolation. Specimens from live animals may include pus, exudates, nasal swab, blood and bronchial lavages (Quinn *et al.*, 1994; Radostits *et al.*, 1994).

*P. multocida* is a facultative anaerobic bacterium that grows best at 35 - 37<sup>0</sup>C. Primarily isolation is usually accomplished by using media such as blood agar, trypticase soya agar, and tryptose soya broth. The organism never grows on MacConkey agar. Recently isolated organisms or those found in tissue smears show bipolar staining with Geimsa or Wright stain, and are usually encapsulated. The cells from media are coccobacillary or short rod shaped. *P. multocida* is a gram negative bacterium. It is catalase and oxidase positive, none motile (OIE, 2004).

*P. multocida* is positive in indole, ornithine decarboxylase; nitrate reduction tests, but negative in urease. It utilizes glucose, sucrose, and mannitol fermentatively, but not lactose and mannose (OIE, 2004).

Biotyping of *P. multocida* is occasionally carried out in epidemiological investigations (Quinn *et al.*, 2002). Three biotypes or subspecies of *P. multocida* are recognized, namely *P. multocida* subspecies *multocida*, *P. multocida* subspecies *septica* and *P. multocida* subspecies *gallicida*. The three subspecies are differentiated by minor differences in their fermentation of carbohydrates. *P. multocida* subspecies *multocida* cause significant disease in domestic animals. It ferments sorbitol, but not dulcitol. It has a variable reaction on trehalose and D-xylose. *P. multocida* subspecies *gallicida* ferments D-xylose, sorbitol and dulcitol, but not trehalose. While *P. multocida* subspecies *septica* ferments only trehalose and D-xylose. The flow chart for isolation and identification of bacteria from poultry samples is presented in figure 1.

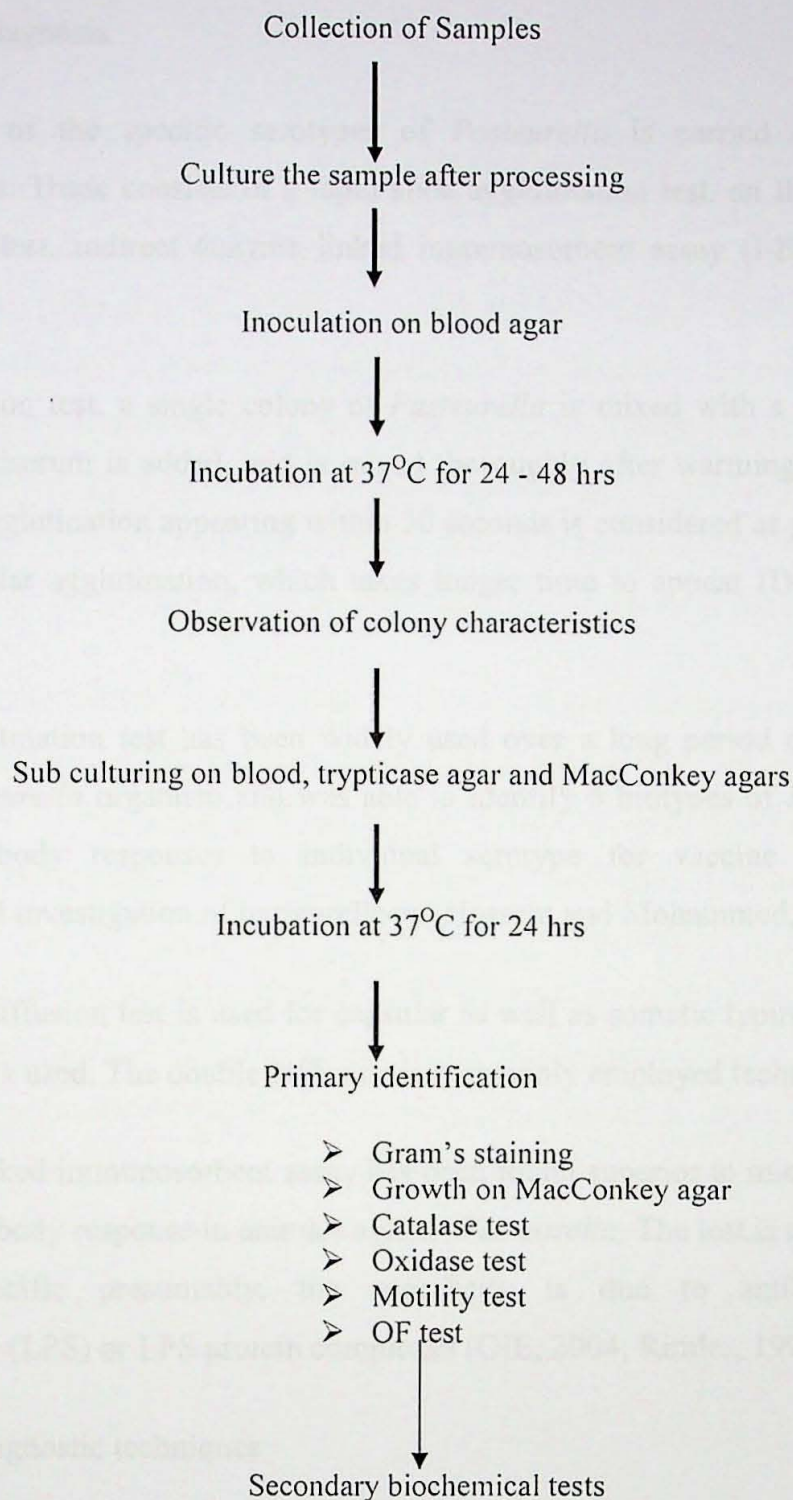


Figure 1: Flow chart for isolation and identification of bacteria from poultry samples

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

### 2.2.3. Serological diagnosis

The identification of the specific serotypes of *Pasteurella* is carried out using different serological methods. These consists of a rapid slide agglutination test, an IHA test for capsular typing, the AGID test, indirect enzyme linked immunosorbent assay (I-ELISA) (OIE, 2004; Rimler, 1993).

In rapid agglutination test, a single colony of *Pasteurella* is mixed with a drop of saline on a slide, a drop of antiserum is added, and is mixed thoroughly after warming the slide gently. A coarse follocular agglutination appearing within 30 seconds is considered as positive. Old culture gives a fine, granular agglutination, which takes longer time to appear (DeAlwis, 1993; OIE, 2004).

Indirect haemagglutination test has been widely used over a long period of time for capsular serotyping of *Pasteurella* organism and was able to identify 5 biotypes of *P. multocida*, and to detect serum antibody responses to individual serotype for vaccine evaluation and in seroepidemiological investigation of pasteurellosis (Hussein and Mohammed, 1984).

Agar gel immune diffusion test is used for capsular as well as somatic typing, depending on the antigens and antisera used. The double diffusion is commonly employed technique (OIE, 2004).

Indirect enzyme linked immunosorbent assay has been found superior to microtiter agglutination for quantifying antibody response in animals against *Pasteurella*. The test is reported to be highly sensitive and specific presumably, the specificity is due to antibodies recognizing lipopolysaccharides (LPS) or LPS protein complexes (OIE, 2004; Rimler, 1993).

### 2.2.4. Molecular diagnostic techniques

Molecular biology has produced techniques that have significantly increased understanding of the epidemiology of fowl cholera. The techniques include DNA finger printing and ribotyping. They are based on unique properties of the bacterial chromosomal DNA, rather than phenotyping expression of the bacteria (Rimler, 1993). A *P. multocida* specific PCR identified all subspecies of *P. multocida* through specific amplification of approximately 460bp DNA fragment (Zeng *et al.*, 1999).

### 2.2.5. Differential diagnosis

Fowl cholera must be differentiated carefully from 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. Fowl cholera can be differentiated readily from most septicemic and viremic diseases of poultry by isolation and identification of *P. multocida*. Fowl cholera always should be suspected if there are epizootic losses in domesticated or wild fowls. Related organisms can cause cholera like diseases or complicate other diseases including *P. gallinarum*, *M. haemolytica*, *Moraxella anatipestifer*, *Moraxella osloensis*, and *Yersinia pseudo tuberculosis*. To differentiate fowl cholera from other diseases, several serological tests have been developed (OIE, 2004). Currently ELISA test is commercially available and widely used.

A small number of outbreaks of pasteurellosis similar to acute or chronic fowl cholera have been reported in chickens and turkeys and were caused by *P. gallinarum* and *M. hemolytica*. These organisms are similar to *P. multocida* in general properties but only a few strains are virulent. The organisms are differentiated biochemically from *P. multocida*. The exact significance of these infections to the poultry industry has yet to be determined (Jordan *et al.*, 1999). 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 widely 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).

### 2.3.1. Prevention

*P. multocida* is not transmitted through the eggs. Therefore, proper control measures may be applied to prevent occurrence of the disease. The following are most important strategies. Chick birds should be obtained and reared in quarantine on disease free premises and away from all other animals that might carry. Birds should not be added to the flock, as they may be carrying bacteria that might cause. Birds should not be added to the flock, as they may be carrying bacteria that might cause. A high standard of sanitation should be practiced. All sick or dead birds should be classified and destroyed to prevent disease spread (Saville, 1994). Carcasses should be disposed by burying or burning to prevent disease spread.

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

| Tests                    | <i>Pasteurella</i> |                       |                  | <i>Riemerella</i>    |
|--------------------------|--------------------|-----------------------|------------------|----------------------|
|                          | <i>multocida</i>   | <i>M. haemolytica</i> | <i>gallicida</i> | <i>anatipestifer</i> |
| Haemolysis on blood agar | -                  | +                     | -                | V                    |
| Growth on MacConkey agar | -                  | +U                    | -                | -                    |
| Indole production        | +                  | -                     | -                | -                    |
| Gelatin liquefaction     | -                  | -                     | -                | +U                   |
| Catalase test            | +                  | +U                    | +                | +                    |
| Urease test              | -                  | -                     | -                | V                    |
| Glucose fermentation     | +                  | +                     | +                | -                    |
| Lactose fermentation     | -U                 | +U                    | -                | -                    |
| Sucrose fermentation     | +                  | +                     | +                | -                    |
| Maltose fermentation     | -U                 | -                     | +                | -                    |
| Ornithine decarboxylase  | +                  | -                     | -                | -                    |

Test reaction results: - = no reaction, + = positive reaction, V = variable reaction, -U = usually no reaction, +U = usually positive

Source: OIE (2004)

## 2.3. Control and prevention

### 2.3.1. Prevention

*P. multocida* is not transmitted through the eggs. Therefore, proper control measures may be applied to prevent occurrence of the disease. The following are most important strategies. Clean birds should be obtained and raised in quarantine on disease free premises and away from all birds and mammals that might carriers. Birds should not be added to the flock, as they may be carriers; stresses should be avoided in so far as is possible, and a high standard of sanitation should be practiced; all sick or dead birds should be identified and destroyed before they can be cannibalized (Saville, 1995). Carcasses should be disposed by burying or burning to prevent them

from being fed on by scavengers (including dogs and cats). Reduce rodents, scavengers and predators in the farm environment and limit their contact with flocks. 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 of old (Arshed, 2002).

Bacterins don't 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 States 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 are frequently given to turkeys at 2 - 4 weeks intervals beginning at 6 - 7 weeks of age in 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 immunosuppression or concurrent diseases. Live vaccine confers better resistance than killed bacterins and offer abroad spectrum of protection against most serotypes (OIE, 2004).

Following an outbreak, depopulation should be considered since many surviving birds become carriers and transmit *P. multocida*. Following depopulation, the premises and equipments 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 vaccination program. Differing susceptibilities among genetic lines of turkey has been shown suggesting that selection for resistance to fowl cholera many be possible (Whiteman and Bickford, 1989).

### 2.3.2. 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 feed 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 include sulfamerazine, sulfadimethoxine, tetracycline, sulfaquinoxaline, sulfamethazine, erythromycin, and streptomycin. Addition of citric acid to drinking water and reduction of calcium or use of a chelating agent in the feed will potentate the effects of the tetracycline. Moving uninfected 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 be effective. If cholera can not 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)

### 3.2. Study animals

The study animals were specific strains of broiler chickens (100-120 days of age) and layers (about 150 days of age) and were kept under strict farm management regime. The flock composition with their management details is shown in Table 2.

### 3. MATERIALS AND METHODS

#### 3.1. Study area

The study was conducted in two randomly selected commercial poultry farms found in Debre Zeit. Debre Zeit is located in Oromia National Regional State about 47 kms South East of Addis Ababa at an altitude of 1850 meters above sea level. It is found at 90°N latitude and 40°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 temperature of 12.3°C and 27.7°C, respectively (CSA, 2006).

#### 3.2. Study animals

The study animals were exotic breeds (*Bovans white*) of growers (60-150 days of age) and layers (above 150 days of age) and all sex groups kept under deep litter management system. The flock population with deep litter management system of the study farms is showed in Table 2.

#### 3.3. Study design

The study was cross sectional with simple random sampling technique to determine the prevalence of *P. multocida* from the upper respiratory tract of apparently healthy birds and to determine the sheep value of *P. multocida* serotype A in the study farms.

In addition, birds in selected commercial poultry farms were followed for 5 months (November 2009 – March 2010). During the follow up period there was no active outbreak of foot cholera in the study farms, but there were 2 layers in farm 2 with typical clinical signs of foot cholera (proliferative, necrotic, ulcer and poly arthritis). Among the lesions, five or more were disseminated air filled, pale swollen air sacs and also appropriate samples were collected and brought to HPAV Microbiology laboratory for bacteriological investigations. During the study period there were no birds which were died from foot cholera in the two commercial poultry farms. All data were kept confidential and subjected for detailed post mortem examination and were kept confidential and were not shared with the district

**Table 2:** The flock population of the two study farms in Debre Zeit.

| Farm code | Breed           | Age in days | Population |
|-----------|-----------------|-------------|------------|
| 1         | <i>B. white</i> | up to 60    | 4728       |
| 1         | <i>B. white</i> | 60-150      | 6200       |
| 1         | <i>B. white</i> | above 150   | 3203       |
| 2         | <i>B. white</i> | up to 60    | 6444       |
| 2         | <i>B. white</i> | 60-150      | 2503       |
| 2         | <i>B. white</i> | above 150   | 7210       |
| Total     |                 |             | 30288      |

Source: 1 and 2 Poultry Farm Records (November, 2009).

### 3.3. Study design

The study was cross sectional with simple random sampling technique to determine the prevalence of *P. multocida* from the upper respiratory tract of apparently healthy fowls and to determine the seroprevalence of *P. multocida* serotype A in study farms.

In addition, fowls in selected commercial poultry farms were followed for 5 months (November 2009 – March 2010). During this follow up period there was no active outbreak of fowl cholera in the study farms, but there were 7 layers, in farm 2 with typical clinical signs of chronic fowl cholera (swollen wattles, combs and poly arthritis). Among the seven, five of them were eviscerated for detailed post mortem examination and also appropriate samples were collected and brought to FVM Microbiology laboratory for bacteriological investigations. During the study period there were 98 fowls which were died from respiratory disease syndrome in the two commercial poultry farms. All these died fowls were eviscerated for detailed post mortem examination and only 35 fowls which had similar macroscopic lesions with the disease,

appropriate samples were collected and brought to FVM Microbiology Laboratory for bacteriological investigations.

### 3.4. Sampling

#### 3.4.1. Sample size

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

$$n = \frac{1.962 \cdot P_{exp} (1 - P_{exp})}{d^2}$$

Where: n = required sample size;  $P_{exp}$  = expected prevalence; d =desired absolute precision

There are no previous studies on prevalence of fowl cholera in chickens in Ethiopia. Therefore, an expected prevalence 50% was used to estimate the sample size. Using desired 95% confidence interval, 5% precision and 50% expected prevalence, the number of fowls needed to detect *P. multocida* and seroprevalence of *P. multocida* serotype A in study farms were 384 birds for each farm.

### 3.5. 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 containers and transported directly in ice box with ice pack to the Microbiology Laboratory of FVM and were kept in refrigerator at 4°C until processed. All samples were clearly labeled with the date of sampling, the type of sample, the farm and flock (OIE, 2004). Samples including, lung, tracheal and nasopharyngeal swabs were collected aseptically for isolation of *P. multocida*. Blood was collected from fowls aseptically using vacutainer tubes to separate serum for indirect haemagglutination test (IHA).

### 3.5.1. Nasopharyngeal swabs

Nasopharyngeal swabs were collected from 384 randomly selected apparently healthy fowls. Before collecting the swabs, the beak of the fowls were well cleaned with cotton wool soaked in 70% ethyl alcohol. Then sterile cotton tipped swabs in screw capped test tube moistened with tryptose soya broth were inserted into the nasopharyngeal region of each fowl, and the mucosa surface was rubbed by rotating the swabs. The swabs were then placed back into 3ml of sterile tryptose soya broth (Oxoid, Hampshire, England) in universal tubes and transported in ice box containing ice packs to the laboratory.

### 3.5.2. Tissue sample collection

The surface of pneumonic lungs was rubbed with cotton soaked in 70% ethanol to minimize surface contaminants. Using sterile materials the part of the lung was cut with scissors held with sterile tongue forceps and put in another sterile screw capped universal bottles. Tracheal swabs were also taken from the same carcass with sterile cotton tipped swabs in screw capped test tubes. Swabs moistened with tryptose soya broth were used to rub the mucosa surface by rotating the swabs. The swabs were placed back into 3ml of sterile tryptose soya broth in universal tubes and transported in ice box containing ice packs to the laboratory (OIE, 2004).

### 3.5.3. Serum Sample Collection

Few feathers were plucked from the ventral surface of the humeral region of the wing, and the site was wiped with cotton wool soaked with 70% ethanol, blood samples for serum separation were collected from wing vein using sterile needles and plain vacutainer tubes with out any anticoagulant. Approximately 5ml of blood sample was collected from each fowl. The vacutainer tubes were labeled and left to stand on inclined position over night at an ambient temperature to allow clotting, and the sera were collected using sterile Pasteur pipette and transferred to sterile cryovials; the cryovials were labeled and stored at  $-20^{\circ}\text{C}$  in deep freezer until the appropriate serological test was performed (OIE, 2004).

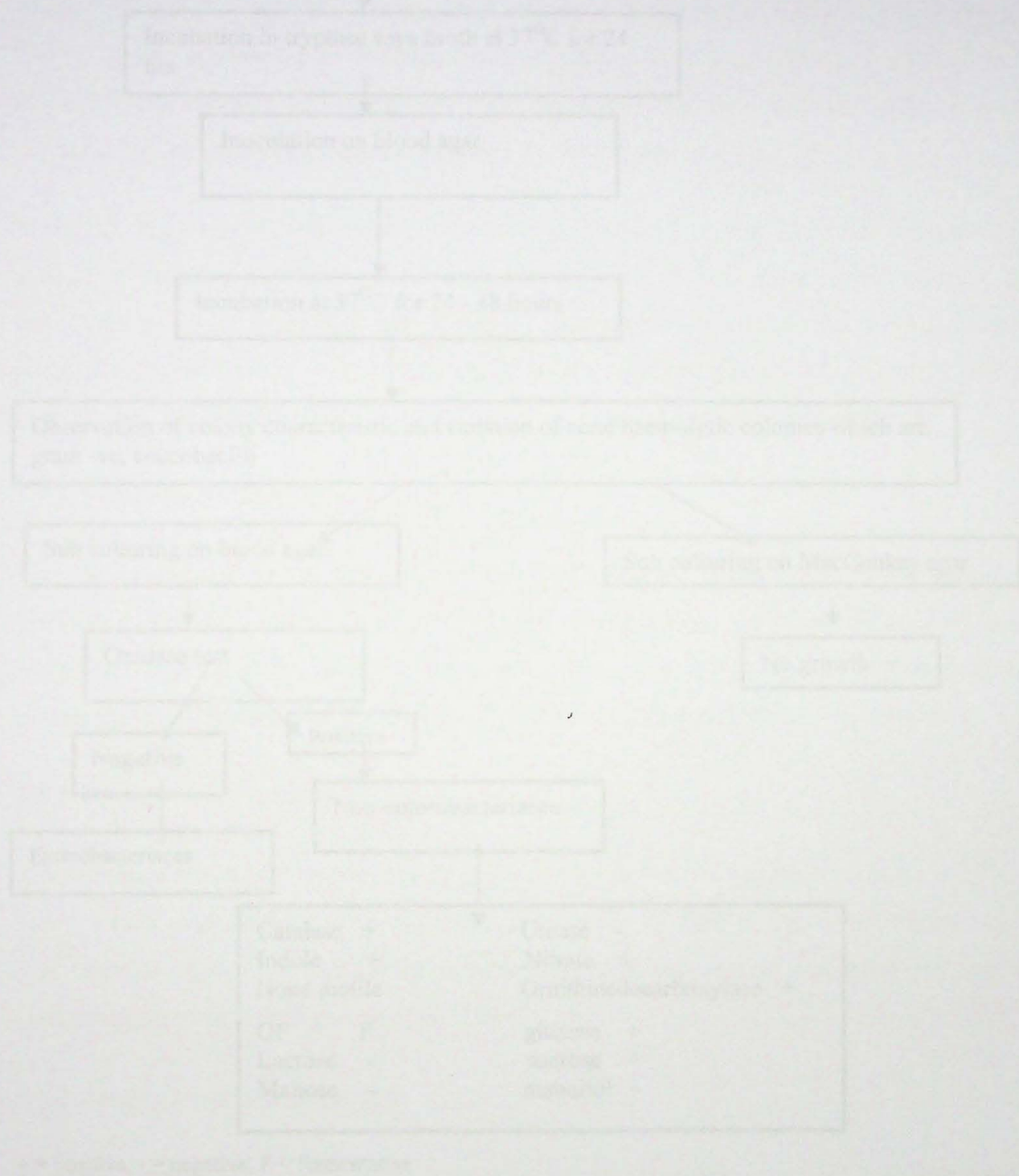
### 3.6. Isolation and identification of *P. multocida*

In the laboratory nasal and tracheal swabs were incubated immediately at 37°C for 24 hours, whereas lung samples were disinfected with 70% alcohol, dried and the samples were cut in to pieces with sterile scissors assisted by sterile tongue forceps and put into 3ml of tryptose soya broth (Oxoid, Hampshire, England) in universal tubes. The universal tubes were tightly closed and incubated at 37°C for 24 hours. After 24 hours of incubation the nasal, tracheal swabs and lung tissues in tryptose soya broth, a loopfull of culture was transferred on to blood agar (Titan Biotech Limited Bhiwadi) containing 7% defibrinated sheep blood, and then streaked by inoculating loop. The plates were incubated at 37°C for 24 hours, and after 24 hours incubation, blood agar plates were examined for the presence or absence and type of haemolysis and general appearance of the colonies including color, shape, size and contour. The colonies suggestive of *P. multocida* were selected and smears were stained with Gram's staining and examined microscopically under oil immersion. Colonies which were gram negative, coccobacilli, short rods with or with out bipolar staining were transferred on to MacConkey and Trypticase soya agar (TSA). Those isolates that were not grown on MacConkey agar after two days of incubation and those not producing haemolysis on blood agar were subjected for oxidase, catalase, and motility and OF test for primary identification. Colonies which were gram negative, coccobacilli or short rods with or with out bipolar staining, oxidase and catalase positive, non motile, indole positive, and fermentative (facultative anaerobe) in OF test were subjected for further biochemical tests to identify the species and subspecies of the organism (OIE, 2004).

After primary characterization of the isolates, secondary biochemical characterization of the organism was performed based on urease, ornithine decarboxylase, and acid and gas production within 24 to 48 hours from glucose, lactose, sucrose, maltose and mannitol. *Pasteurella multocida* was selected on the basis of indole production, nitrate reduction, characteristics of sweetish odor on culture plates, absence of growth and haemolysis on MacConkey and blood agar, respectively; and fermentation of glucose, sucrose and mannitol with out gas production but not lactose and maltose (Quinn *et al.*, 2002).

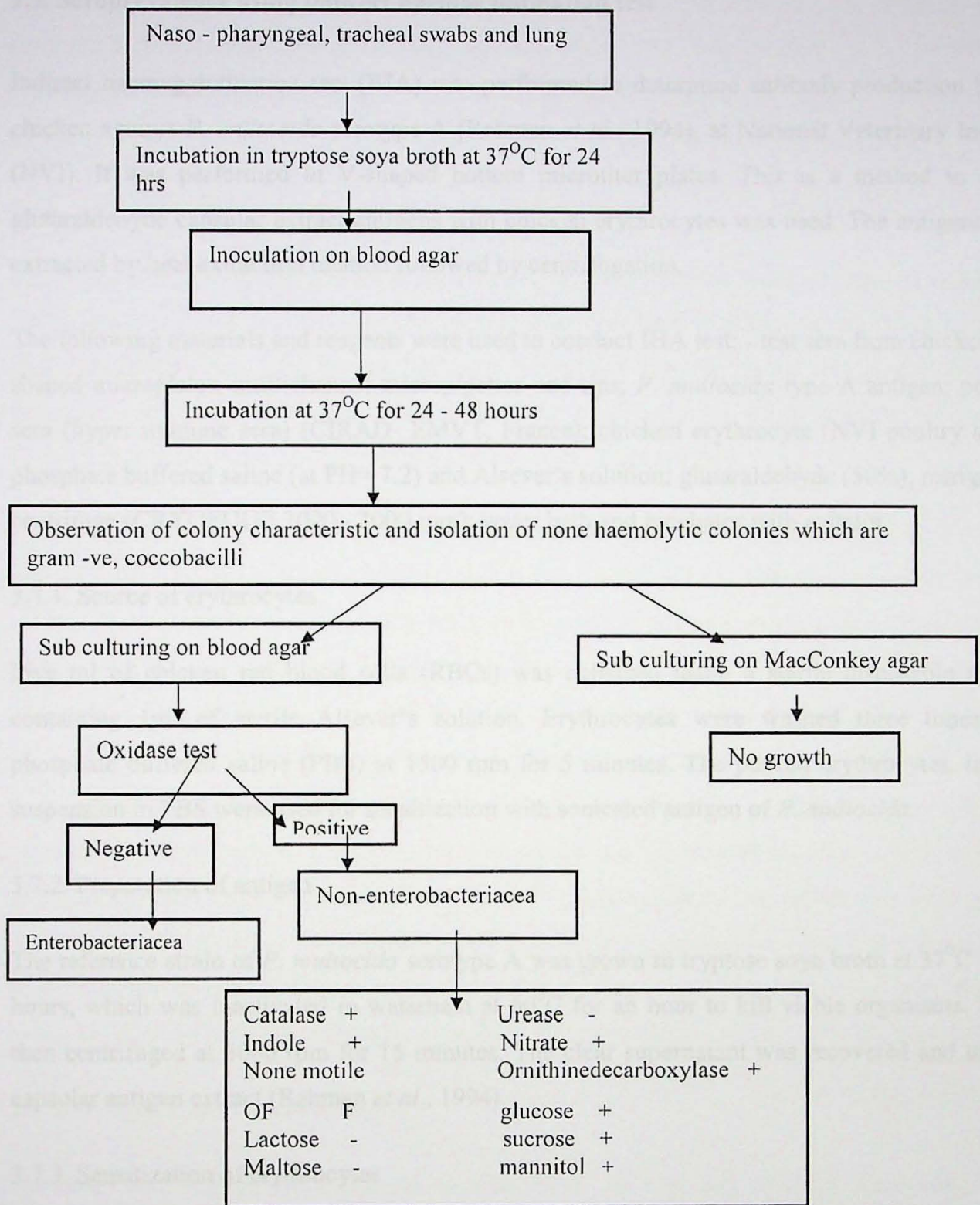
Characterization of the isolates up to subspecies level was performed based on fermentation of trehalose, D-xylose, L-arabinose, sorbitol and dulcitol. *P. multocida* subspecies *gallicida* was

selected on the basis of acid production but not gas from D-xylose, sorbitol and dulcitol, where as *P. multocida* subspecies *septica* produced acid from trehalose and D-xylose only (Quinn *et al.*, 2002). The flow chart for isolation and identification of *P. multocida* is showed in figure 2.



Source: (Quinn, 2004)

Figure 2.1 Flow chart for isolation and identification of *P. multocida*



+ = positive, - = negative, F = fermentative

Source (OIE, 2004)

**Figure 2:** Flow chart for isolation and identification of *P. multocida*

### 3.7. Seroprevalence using indirect haemagglutination test

Indirect haemagglutination test (IHA) was performed to determine antibody production by the chicken against *P. multocida* serotype A (Rahman *et al.*, 1994), at National Veterinary Institute (NVI). It was performed in V-shaped bottom microtiter plates. This is a method in which glutaraldehyde capsular extract antigens with chicken erythrocytes was used. The antigens were extracted by heat extraction method followed by centrifugation.

The following materials and reagents were used to conduct IHA test: - test sera from chickens; V-shaped microplates, multichannel micropipettes and tips; *P. multocida* type A antigen; positive sera (hyper immune sera) (CIRAD- EMVT, France); chicken erythrocyte (NVI poultry stock); phosphate buffered saline (at PH= 7.2) and Alsever's solution; glutaraldehyde (50%); refrigerated centrifuge (CRYOFUGE 2000 - 3000 rpm); water bath and incubator with agitator.

#### 3.7.1. Source of erythrocytes

Five ml of chicken red blood cells (RBCs) was collected using a sterile disposable syringe containing 1ml of sterile Alsever's solution. Erythrocytes were washed three times with phosphate buffered saline (PBS) at 1500 rpm for 5 minutes. The packed erythrocytes, in 10% suspension in PBS were used for sensitization with sonicated antigen of *P. multocida*.

#### 3.7.2. Preparation of antigen

The reference strain of *P. multocida* serotype A was grown in tryptose soya broth at 37°C for 24 hours, which was inactivated in waterbath at 60°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 capsular antigen extract (Rahman *et al.*, 1994).

#### 3.7.3. Sensitization of erythrocytes

This was performed stepwise as follows. Chicken erythrocytes (from NVI poultry farm) were freshly collected in Alsever's solution in a ratio of 3 to 8 by volume. The erythrocytes were washed three times in PBS by centrifugation at 2000rpm for 15 minutes. One hundred µl packed RBCs, were added to 10 ml of each antigen. Fifty µl of 50% glutaraldehyde was added and

mixed by gentle shaking. The mixture was incubated for one hour at 37°C with periodical shaking, and centrifuged at 2000rpm for 10 minutes and then washed twice in PBS by centrifugation. Finally 10ml of PBS was added to the final sediment and a 1% RBC solution was made.

#### 3.7.4. Test procedure

Indirect haemagglutination antibody titers of all the sera samples were determined against *P. multocida* serotype A by microtitration technique as described (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 sera samples column wise simultaneously. The last 2 columns (column 11 and 12) of wells were left for positive and negative serum controls.

All the wells were dispensed with 50µl PBS using multichannel micro dispenser leaving the first well of each row empty in which 100µl test sera 1/10 diluted in PBS were added to the first column of the plate. Samples were placed up to column 10. All sera samples were diluted by two fold serial dilution (1/20, 1/40 and up to 1/320) in PBS with the help of multichannel micro dispenser. Using multichannel pipette, 50µl of sensitized erythrocytes were added into each well except the wells of the last column of the plates, which were used for negative control. The plates were gently shaken backward and forward and also from side to side to ensure an even suspension of erythrocytes. Then, they were incubated at 37°C for one hour. The dilution of each serum sample causing complete or more than 50% haemagglutination pattern was taken as end point. The titer showing 1/40 dilution and above were taken as positive. The IHA antibody titer was displayed as the reciprocal of its end point dilution. The IHA titer of all sera samples obtained were recorded and geometric means of titers were calculated and analyzed statistically.

#### 3.8. Data management and analysis

The data were entered and managed in MS Excel. All the data analyses were done using Statistical Package for Social Sciences (SPSS) Software version 15. Descriptive statistics such as percentages and frequency distributions were used to describe the nature and the characteristics of data. Prevalence of *P. multocida* isolates was analyzed using percentages. Seroprevalence was analyzed using percentages and geometric mean. The association of different factors with

isolation of *P. multocida* and in seroprevalence was computed by Chi-square ( $\chi^2$ ) test. Strength of association between risk factors in seropositivity was computed by using univariable logistic regression.

## 4. RESULTS

### 4.1. Cellular morphology

*Pasteurella multocida* isolates revealed different morphological features on smears made from fresh and cultures. The isolates were gram negative, short ovoid rods with an occasional tendency to bipolar staining. Cells arranged in chain were also observed. After repeated culture on agar, the organism tends to form longer rods and to become more pleomorphic, forming chains, filaments, and rods of various sizes.

### 4. 2. Cultural characteristics

*Pasteurella multocida* revealed round, grayish, and non hemolytic occasionally mucoid colonies with a characteristic “Sweetish” odor. None of *P. multocida* isolates showed growth on MacConkey agar after 48 hours of incubation. The colonies of subcultures on blood agar were small and non mucoid. This is because the bacterium losses capsule in subcultures.

### 4. 3. Identification using biochemical tests

A total of 82 suspected isolates collected from nasal, tracheal swabs, and pneumonic lungs were identified using biochemical tests. All isolates were positive for indole, nitrate reduction and ornithine decarboxylase tests, but negative for urease and H<sub>2</sub>S production. All the isolates were able to utilize glucose, sucrose and mannitol fermentatively. A total of 62 isolates were positive for utilization of D-xylose, sorbitol and dulcitol; but negative for utilization of trehalose and L-arabinose. Based on this biochemical test results the isolates were identified as *P. multocida* subspecies *gallicida*. The remaining 20 isolates were positive for utilization of trehalose and D-xylose, but negative for L-arabinose, sorbitol and dulcitol. Thus, they were identified as *P. multocida* subspecies *septica*.

#### 4. 4. Bacteriological findings

A total of 82 samples out of 489 were positive for *P. multocida* (Table 3). The percentage rate of recovery of *P. multocida* varied from the nasal swabs of apparently healthy fowls (14.58%), nasal swabs of diseased fowls with respiratory disease syndrome (25.71%), tracheal swabs of died fowls at respiratory disease syndrome (17.14%), pneumonic lungs of died fowls at respiratory disease syndrome (26.67%) and pneumonic lungs of fowls with typical clinical signs of chronic fowl cholera (60.00%). The overall isolation rate of *P. multocida* from nasal swabs, tracheal swabs and pneumonic lungs were 16.76%. There was statistically significant difference ( $P < 0.05$ ) between the isolation rate of *P. multocida* from nasal swabs of apparently healthy fowls and fowls showing respiratory disease syndrome as indicated in Table 4.

**Table 3:** Prevalence of *P. multocida* isolates from respiratory tracts of apparently healthy fowls, fowls diseased with respiratory disease syndrome and tentatively diagnosed cases of chronic fowl cholera.

| Samples                  | No of samples | Positive    | 95% CI of prevalence |
|--------------------------|---------------|-------------|----------------------|
| Nasal swabs from ahf     | 384           | 56 (14.58%) | 5.34 - 23.82         |
| Nasal swabs from dsfr    | 35            | 9 (25.71%)  | 11.24 - 40.18        |
| Tracheal swabs from difr | 35            | 6 (17.14%)  | 4.58 - 29.70         |
| Lung from difr           | 30            | 8 (26.67%)  | 10.85 - 42.49        |
| Lung from difcfc.        | 5             | 3 (60.00%)  | 17.06 - 102.94       |
| Total                    | 489           | 82 (16.76%) | 13.45 - 20.07        |

\*ahf = apparently healthy fowls; dsfr = diseased fowls with respiratory disease syndrome; difr. = died fowls from respiratory disease syndrome; difcfc = died fowls with chronic fowl cholera.

**Table 4:** Association of the isolation rate of *P. multocida* from nasal swabs of apparently healthy fowls and fowls diseased with respiratory syndrome

| Samples                                   | Positives | Prevalence | 95%CI         | $\chi^2$ | P-value |
|-------------------------------------------|-----------|------------|---------------|----------|---------|
| Nasal swabs from apparently healthy fowls | 56        | 14.58%     | 5.33 - 23.82  | 11.3     | 0.018   |
| Nasal swabs from diseased fowls           | 9         | 25.71%     | 11.24 - 40.18 |          |         |

Furthermore, the total isolates of *P. multocida* were identified at subspecies level using the method given in Quinn *et al.* (2002). Accordingly, in apparently healthy fowls there were 39 (69.64%) and 17 (30.36%) *P. multocida* subspecies *gallicida* and *septica*, respectively. Whereas in fowls diseased with respiratory syndrome, there were 23 (88.46%) and 3 (11.54%) *P. multocida* subspecies *gallicida* and *septica* respectively, as shown in Table 5.

**Table 5:** Distribution of *P. multocida* subspecies isolates in apparently healthy fowls and fowls diseased with respiratory syndrome.

|                   |                | Isolated sub species                 |                                    |
|-------------------|----------------|--------------------------------------|------------------------------------|
| Status of fowls   | No of isolates | <i>P.m</i> sub spp. <i>gallicida</i> | <i>P.m</i> sub spp. <i>septica</i> |
| Healthy           | 56             | 39 (69.64%)                          | 17 (30.36%)                        |
| Diseased and died | 26             | 23 (88.46%)                          | 3 (11.54%)                         |
| Total             | 82             | 62 (75.61%)                          | 20 (24.39%)                        |

*P.m* sub spp. = *Pasteurella multocida* sub species

For the purpose of understanding, if there was difference in isolation rate of *P. multocida* in different age groups died with respiratory disease syndrome, their pneumonic lungs and corresponding tracheal swabs of layers and growers were examined from the two commercial

poultry farms in study period. Fourteen and sixteen pneumonic lungs and their corresponding tracheal swabs of growers and layers respectively were sampled and examined. The association and isolation rate of *P. multocida* from pneumonic lungs and corresponding tracheal swabs of different age groups of fowls are showed in Table 6 and 7. It was observed that the isolation rate of *P. multocida* from pneumonic lungs and tracheal swabs were higher in layers (37.5%) and (18.75%) and lower in growers (14.2%) and (7.14), respectively. There was statistically significant difference ( $P<0.001$ ) between the isolation rate of *P. multocida* between the two age groups in the study farms.

**Table 6:** Association of the isolation rate of *P. multocida* from pneumonic lungs of the two age groups of fowls with respiratory disease syndrome.

| Age of fowls | Samples | Sampled | Prevalence (%) | 95% of CI     | $\chi^2$ | p-value |
|--------------|---------|---------|----------------|---------------|----------|---------|
| Grower       | lung    | 14      | 14.28          | 4.08 - 32.48  | 17.08    | 0.000   |
| Layer        | lung    | 16      | 37.50          | 13.78 - 61.22 |          |         |

**Table 7:** Association of the isolation rate of *P. multocida* from tracheal swabs of the two age groups of fowls with respiratory disease syndrome.

| Age of fowls | Samples       | Sampled | Prevalence (%) | 95%CI        | $\chi^2$ | p-value |
|--------------|---------------|---------|----------------|--------------|----------|---------|
| Grower       | Tracheal swab | 14      | 7.14           | 6.34 - 20.62 | 16.08    | 0.000   |
| Layer        | Tracheal swab | 16      | 18.75          | 0.5 - 37.87  |          |         |

#### 4. 5. Seroprevalence of *P. multocida*

A total of 384 sera were collected from chicken in the study area. The presence of antibodies specific to *P. multocida* serotype A in chicken sera was studied using IHA. Accordingly, out of the 384 sera 19.01% were positive for *P. multocida* serotype A capsular antigen. Due to lack of reference anti sera specific to capsular antigen of other serotypes, the seroprevalence of the other *P. multocida* serotypes could not be determined. The seroprevalence of *P. multocida* serotype A in two farms is presented in Table 8.

**Table 8:** Seroprevalence of *P. multocida* serotype A in two farms

| Farm code | Sampled | Positive    | 95% of CI     |
|-----------|---------|-------------|---------------|
| 1         | 186     | 16 (8.60%)  | 4.58 - 12.62  |
| 2         | 198     | 57 (28.78%) | 22.48 - 35.08 |
| Total     | 384     | 73 (19.01%) | 15.09 - 22.93 |

**Table 9:** Distribution of titration value of positive chicken sera by indirect haemagglutination test (IHAT)

| Age                 | Titration |      |       |       | Geo mean | STD     |
|---------------------|-----------|------|-------|-------|----------|---------|
|                     | 1/40      | 1/80 | 1/160 | 1/320 |          |         |
| Grower(2-5months)   | 10        | 2    | 3     | 2     | 1/67     | 0.00887 |
| Layer (> 5 months ) | 13        | 13   | 19    | 11    | 1/114    | 0.00817 |
| Total               | 23        | 15   | 22    | 13    | 1/100    | 0.00879 |

\*Geo mean = Geometric mean; STD. = Standard deviation

The seroprevalence ranges from 8.60% to 28.78% in two farms and the overall seroprevalence of the two farms was 19.01%. The seroprevalence of fowl cholera in the study farms was different by age groups, which was 9.65% and 26.92% for growers and layers, respectively. The highest seroprevalence was recorded in layers (26.92%) followed by growers (9.65%). The seroprevalence of fowl cholera by different age groups is presented in Table 10.

**Table 10:** Seroprevalence of *P. multocida* serotype A by different age groups.

| Age                   | Sampled | Positives   | 95% CI of prevalence |
|-----------------------|---------|-------------|----------------------|
| Growers (2 - 5months) | 176     | 17 (9.65%)  | 5.29 - 14.01         |
| Layers(> 5 months)    | 208     | 56 (26.92%) | 20.90 - 32.94        |
| Total                 | 384     | 73 (19.01%) | 15.09 - 22.93        |

There was statistically significant difference ( $P < 0.01$ ) in seroprevalence of *P. multocida* serotype A between the two farms and age groups as shown in Table 11. The seroprevalence was higher in farm 2 (28.78%) than in farm 1 (8.60%) as shown in Table 11.

**Table 11:** Association of risk factors in seropositivity.

| Factors | Farm code | Sampled | Positives   | $\chi^2$ | P-value |
|---------|-----------|---------|-------------|----------|---------|
| Farm    | 1         | 186     | 16 (8.60%)  | 25.38    | 0.00    |
|         | 2         | 198     | 57 (28.78%) |          |         |
| Age     | Grower    | 176     | 17 (9.65%)  | 18.45    | 0.00    |
|         | Layer     | 208     | 56 (26.92%) |          |         |

Strength of association between risk factors in seroprevalence of *P. multocida* serotype A was computed using univariable logistic regression as indicated in Table 12. From the result of odds ratio (OR) we can conclude that layers were 3.446 times at risk of suffering from fowl cholera than growers. While chicken in farm 2 were 3.18 times at risk of suffering from fowl cholera than those in farm 1.

**Table 12:** Strength of the association between risk factors in seropositivity

| Risk factors | Farm code | Sampled | Prevalence  | P-value | OR    | 95% CI of OR  |
|--------------|-----------|---------|-------------|---------|-------|---------------|
| Farm         | 1         | 186     | 16 (8.60%)  | 0.000   | 3.181 | 1.683 - 6.014 |
|              | 2         | 198     | 57 (28.78%) |         |       |               |
| Age          | Grower    | 176     | 17 (9.65%)  | 0.000   | 3.446 | 1.917 - 6.195 |
|              | Layer     | 208     | 56 (26.92%) |         |       |               |

## 5. DISCUSSION

Fowl cholera is a bacterial disease of domestic and other fowls. This disease results 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 apparently healthy fowls, fowls diseased with respiratory disease syndrome and tentatively diagnosed cases of fowl cholera in two commercial poultry farms in Debre Zeit. All isolates were identified based on their cultural, morphological and biochemical characteristics. None of the isolates was able to grow on MacConkey agar. The colony characteristics were best studied on blood agar, where the growth was characterized as small grayish colonies without any evidence of haemolysis. Similar results have been previously reported by many authors (Heddleson *et al.*, 1964; Rhoades and Rimler, 1991; Dividedi 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). Motility studies of the isolates on SIM media indicated that all of them were non motile. Results of morphological, staining, colony, cultural and biochemical activities were in total agreement with those documented by Merchant and Packer (1983), Carter *et al.* (1984) and Quinn *et al.* (2002). Accordingly, the isolates of *P. multocida* were positive for oxidase, catalase, indole production, nitrate reduction and ornithine decarboxylase tests. But they were negative for urease and H<sub>2</sub>S production in TSI agar slant Quinn *et al.* (2002). Results of sugar fermentation were also in agreement with previous finding (Cruickshank, 1975; Liowa and Toe, 1978; Rhoads and Rimler, 1991). All isolates utilized glucose, sucrose, and mannitol fermentatively, but, not lactose and maltose.

The isolates of *P. multocida* subspecies *gallicida* utilized D-xylose, sorbitol, and dulcitol fermentatively, but not trehalose; while *P. multocida* subspecies *septica* utilized trehalose and D-xylose. The results were in agreement with those described by Quinn *et al.* (2002).

In the present study, *P. multocida* was isolated at the rate of 14.58% and 25.71% from nasal swabs of apparently healthy fowls and fowls with respiratory disease syndrome, respectively. While 17.14% and 26.67% were isolated from tracheal swabs and pneumonic lungs of fowls died of respiratory disease syndrome, respectively. The organism was isolated at the rate of 60.00%

from pneumonic lungs of fowls with clinical signs of chronic fowl cholera. Isolation of *P. multocida* from pneumonic lungs may indicate that the organism is important in the induction of pneumonic fowl cholera in the study farms (Carter and Subrouto, 1975).

The isolation rate of *P. multocida* from nasal swabs of apparently healthy fowls and fowls with respiratory disease syndrome was 14.58% and 25.71%, respectively. The difference was statistically significant ( $P<0.05$ ). This could be due to the fact that the organism is normal inhabitant of the upper respiratory tract of fowls and invasion to the lower tissue (lung) of diseased fowls, when the immune system of the animals is compromised by different factors (Lopez, 1995). The bacteria multiply in the upper respiratory tract and serves as a source of infection to the lower respiratory tract in diseased fowls. Pneumonia associated with this bacterium is recorded as primary disease in fowls elsewhere (Shewan, 1986; Gilmour *et al.*, 1993).

The isolation rate of the organism from tracheal swabs and pneumonic lungs of fowls died of respiratory disease syndrome were different by age groups, which was higher in layers (18.75% and 37.50%) than in growers (7.14% and 14.28%), respectively. This difference was statistically significant ( $P<0.01$ ). This could be due to the higher susceptibility of fowls with age of 25 weeks and above to the organism than younger chickens, which was in total agreement with the work of Yong - Ku and Kim (2006) in Korea. Although the reasons behind this difference between age groups, especially the higher isolation rate in older age group, are not clear, colonization of the respiratory tract with the bacteria may increase with the age of the animal.

The serological test results showed that the overall seroprevalence of *P. multocida* serotype A in study farms was 19.01%, which was different by age groups and farms. The higher seroprevalence was recorded in farm 2 (28.78%) compared to the farm 1 (8.60%). This difference was statistically significant ( $P<0.001$ ). This could be due to the difference in proper utilization of veterinary drugs and vaccines as prophylactic measures to control many other poultry diseases as part of disease management practice in the farm and difference in biosecurity measures and husbandry practices, which were worse in farm 2. The seroprevalence was also, different by age groups, which was higher in layers (26.92%) than in growers (9.65%). This difference was statistically significant ( $P<0.001$ ). This could be due to fowls at the age of 25 weeks and above

are more susceptible to the disease than younger chickens, and was in total agreement with previous reports (Carter, 1984; Yong - ku and Kim 2006). But the seroprevalence of *P. multocida* serotype A in broiler intensive farms was found to be 8.5% in Korea (Yong-ku and Kim, 2006; Michael, 2005). This could be due to the difference in breed type, time of blood collection following infection, difference in management, husbandry practice, and biosecurity measures.

*P. multocida* is a serious pathogenic disease of domestic and wild birds and considered an important threat for the poultry industry due to high mortality and morbidity. In Ethiopia, even though the disease causes high economic losses in both organic and backyard poultry production systems, due to high morbidity, mortality, loss in production and high treatment cost, the prevalence of the disease was not known. With this anxiety, this study tried to isolate and identify the organism from apparently healthy broilers, diagnosed birds with respiratory distress, diarrhoea and in lambs with respiratory distress. In addition serological test was performed to determine the seroprevalence of *P. multocida* serotype A in study farms. Both bacterial and serological test result of this study showed that the causal agent of fowl cholera is prevalent in the study farms.

In view of this conclusion the following outlooks are forwarded

- There have been economic losses due to fowl cholera in intensive, semi intensive and extensive poultry production systems in Ethiopia. But the prevalence of the disease has not been studied yet in backyard, semi intensive and intensive poultry farms of different management practices. So the prevalence of the disease will be studied further to take appropriate measures.
- Although inactivated fowl cholera vaccines have been produced in other countries, the poultry enterprises in Ethiopia continue to witness reports of continuous losses due to unavailability of cost effective vaccine. To alleviate this problem, effort should be made to produce effective vaccine in our country.
- Due to unavailability of the reference strain and also, the serotype identification of the isolates has not been made. Thus, future studies have to be done to identify serotypes. This would help in developing effective vaccine.

## 6. CONCLUSION AND RECOMMENDATIONS

Poor husbandry practice, low productive potential of local breeds, 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 septicemic 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 disease causes high economic losses in state, private and backyard poultry production systems, due to high morbidity, mortality, loss of production and high treatment cost, the prevalence of the disease was not known. With this anxiety, this study tried to isolate and characterized the organism from apparently healthy fowls, diseased fowls with respiratory disease syndrome and in tentatively diagnosed cases of fowl cholera. In addition serological test was performed to determine the seroprevalence of *P. multocida* serotype A in study farms. Both bacterial and serological tests result of this study showed that the causal agent of fowl cholera is prevalent in the study farms.

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- 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 effort should be made to produce effective vaccine in our country.
- Due to unavailability of the reference strain and sera, the serotype identification of the isolates has not been made. Thus, future studies must be done to identify serotypes. This would help in designing effective vaccine.

- Appropriate biosecurity and sanitation measures must be applied in intensive commercial poultry farms.

- Ashenafi, H. (1995). Avian pathology of industrial poultry farms in Ethiopia. In: IAL (ed.). *1994 national livestock management conference (NLMC)*. Addis Ababa, Ethiopia, January 11-13, 1995. Pp 144-147.
- Ashenafi, H. (1995). Poultry production in Ethiopia. *World Poul. Sci. J.* 51: 191-201.
- Ashenafi, H. (2001). Studies on common pathogens of local chickens. PhD thesis on Veterinary Microbiology, University of Agriculture Faisalabad, Faculty of Veterinary Science, Pakistan.
- Ashenafi, H. (2002). Survey of identification of major diseases of local chickens in some selected agro-ecological zones in central Ethiopia. Faculty of Veterinary Medicine, Addis Ababa University, DVM Thesis.
- Ashenafi, H. and Samra, Y. (2004). Study on major infectious diseases of local chickens in central Ethiopia. *Av. Res. Med.* 159: 301-307.
- Barnes, J., Carter, G., Reid, W. (1964). Diseases of poultry. 5<sup>th</sup> ed. Iowa, USA: Iowa state University press. Pp 141-149.
- Celik, B., Reid, W., Yalci, H. (2004). Diseases of poultry. 6<sup>th</sup> ed. Iowa, USA: Iowa state University press. Pp 209-212.
- Carter, G. and Schmidt, P. (1975). Identification of type A strain of *P. multocida* using serological techniques. *Proc. Soc.* 13: 131-133.
- Carter, G. (1972). Improved serotyping technique for identifying type A strain of *P. multocida*. *Appl. Microbiol. Biotechnol.* 24:162-163.
- Carter, G. (1984). *Parasitology, Parasites and Protozoa*, for Students of Veterinary Bacteriology and Mycology, 4<sup>th</sup> ed. Lee and Heiger, Philadelphia, London. Pp 170-174.

## 7. REFERENCES

- Alamargot, J. (1987): Avian pathology of industrial poultry farms in Ethiopia. In: IAR (ED.): First national livestock improvement conference (NLIC), Addis Ababa, Ethiopia, February 11-13, 1987, Pp 144 - 147.
- Alemu, Y. (1995): Poultry production in Ethiopia. *World. Poul. Sci. J.*, **51**: 197 - 201.
- Arshed, M. (2002): Studies on immuno prophylaxis of fowl cholera, PhD thesis on Veterinary Microbiology, University of Agriculture Faisalabad, Faculty of Veterinary Science, Pakistan.
- Ashenafi, H. (2000): Survey of identification of major diseases of local chickens in three selected agro climatic zones in central Ethiopia. Faculty of Veterinary Medicine, Addis Ababa University, DVM Thesis.
- Ashenafi, H. and Eshetu, Y. (2004): Study on gastro intestinal helminthes of local chickens in central Ethiopia. *Rev. Vet. Med.*, **155**: 504 - 507.
- Barnes, H., Calnek, B., Reid, W. (1998): Diseases of poultry. 8<sup>th</sup> ed. Iowa, USA: Iowa state University press, Pp 141 - 160.
- Calnek, B., Reid, W., Yoder, H. (2001): Diseases of poultry. 6<sup>th</sup> ed. Iowa, USA: Iowa state University press, Pp 260 - 282.
- Carter, G. and Subrouto. P. (1975): Identification of type A strain of *P. multocida* using staphylococcal hyaluronidase. *Vet. Rec.*, **12**: 132 - 135.
- Carter, G. (1972): Improved haemagglutination test for identifying type A strain of *P. multocida*. *Appl. Microbiol. Biotechnol.* **24**:162 - 163.
- Carter, G. (1984): *Pasteurella*, *Yersinia* and *Francisella*. In: Essentials of Veterinary Bacteriology and Mycology. 4<sup>th</sup> ed. Lea and Beiger, Philadelphia, London, Pp 170 - 178.

- Cruickshank, R. (1975): Medical Microbiology. Vol. 11, 12<sup>th</sup> ed, Churchill Livingstone, Edinburgh, London, New York. Pp 456 - 600.
- CSA (2006): Ethiopia agricultural sample enumeration. Central Statistical Authority of the Federal Democratic Republic of Ethiopia, Addis Ababa, 2:48 - 50.
- DeAlwis, M. (1993): Pasteurellosis in production animals. In: Review of ACIR. Pattern, B. E., Spencer, T. L. Jonsson, and R. (Eds) Australia, Canberra, Pp 11 - 12.
- Dessie, T. and Ogle, B. (2001): Village poultry production systems in the central highlands of Ethiopia. *Trop. Anim. Hlth. Produ.*, **33**: 521 - 537.
- Dividedi, P. and Sodhi, S. (1998): Morphological, cultural and biochemical characterization of *P. multocida* isolated from poultry. *Poul. Adv.*, **22**:69 - 71.
- Dung, W. (1992): The respiratory system, In: Jubb K., Kenney P., Palmer N. (Eds.), Pathology of domestic animals. 4<sup>th</sup> ed, Academic press, San Diego, Pp 589 - 663.
- Edwards, A. (1996): Respiratory disease of feedlot cattle in the central USA, *Bovine Pract.*, **30**: 5 - 7.
- Gilmour, N., Pattern, B., Townsend, K., Dawkins, H. and Papa dimitriou, J. (1993): REP - PCR analysis of *Pasteurella multocida* isolates that cause hemorrhagic septicemia. *Res. Vet. Sci.*, **63**: 151 - 155.
- Heddleston, K. (1962): Studies on pasteurellosis. V: Two immunogenic types of *P. multocida* associated with fowl cholera. *Avian Dis.*, **6**: 315 - 321.
- Heddleston, K., Watko, L. and Reoers, P. (1964): Dissociation of a fowl cholera strains of *P. multocida*. *Avian Dis.*, **8**: 649 - 657.
- Heddleston, K., Gallaher, J. and Rebers, P. (1972): Fowl cholera: Gel diffusion precipitin test for serotyping *P. multocida* from avian species. *Avian Dis.*, **16**: 925 - 936.

- Horst, P. (1989): Achievements, difficulties and future prospects in the small scale poultry development. Institute for Animal production, Technical University of Berlin, Germany, PhD Thesis.
- Hussein, A. and Mohammed, E. (1984): Serology in *Mycoplasma*. *Vet. Sci.*, **47**: 84 - 89.
- Jordan, F., Pasttison, M., Alexander, D., Faragher, and T. (1999): Poultry diseases. 5<sup>th</sup> ed. London, England: Harcourt press, Pp 131 - 137.
- Kitalyi, A. (1997): Village Chicken Production systems in developing countries. What does the future hold? *World Anim. Rev.*, **89**: 48 - 53.
- Liowa, T. and Teo, T. (1978): Biochemical studies on *P. multocida* isolated from pigs and poultry. *Sing. Vet. J.*, **2**: 29-32.
- Lopez, A. (1995): Respiratory system. Thomson's, Special Veterinary Pathology. 2<sup>nd</sup> ed. Mosby, London, Pp 116 - 174.
- Lopez, A. (2001): Respiratory systems, thoracic cavity and pleura, In: McGavin M., Carlton W., Zachary J. (Eds), Thomson's special veterinary pathology. 3<sup>rd</sup> ed., Mosby, Saint-Louis, Pp 125 - 196.
- Merchant, I. and Packer, R. (1983): The genus of *Pasteurella*. In: Veterinary Bacteriology and Virology, 7<sup>th</sup> ed., CBS Publisher, New Delhi, Pp 341 - 343.
- Michael, D. (2005): Avian cholera in water fowls. *Wild Life Dis. J.*, **41**: 48 - 57.
- OIE (2004): Diagnosis of Pasteurellosis. In: Manual of standards for diagnostic test and vaccine in terrestrial animals, **2**: 1 - 27.
- Quinn, P., Carter, M., Markey, B. and Carter, G. (1994): *Pasteurella* species. In: Clinical Veterinary Microbiology. Wolfe publishing. Mosby, London, Pp 254 - 258.
- Quinn, P., Morkey, B., Carter, M., Donnelly, W., Leonard, F. and Maguire, D. (2002): *Pasteurella* species and *Mannheimia haemolytica*. In: Veterinary Microbiology and

Microbial diseases. Black well publishing company: Black well science London, Pp 137 - 142.

- Radostits, O., Blood, D., Gay, C. (1994): Disease caused by *Pasteurella* species. In: Veterinary medicine, Text book of the disease of cattle, sheep, pigs, goats and horses. 9<sup>th</sup> ed. Bailliere Tindall, London, Pp 770 – 774.
- Radostits, O., Gray, C., Blood, D. and Hinchcliff, K. (2000): Veterinary medicine. Text book of the disease of cattle, sheep, pigs, goats and horses. 10<sup>th</sup> ed. Harcourt publishers limited, London, Philadelphia, Pp 701 - 867.
- Rahman, S., Ashfaq, M., Rahman, Z. and Hur, G. (1994): *P. multocida* Robert type-1 antibodies in camels. *Pak. Vet. J.*, **14**: 197 - 199.
- Randall, C. (1991): Disease of domestic fowl and turkey. 2<sup>nd</sup> ed. London, England: Wolf press, Pp 16 - 20.
- Rhodes, K. and Rimler, R. (1991): Fowl cholera pathogenesis. *Avian Dis.*, **34**: 381.
- Rimler, R. (1993): Laboratory techniques for *Pasteurella* typing and diagnosis of infections. Australia, Canberra, Pp 136 - 200.
- Rimler, R. (1994): Presumptive identification of *P. multocida* serogroups A, D, and F by capsule depolymerisation with mucopolysaccharides. *Vet. Rec.*, **134**: 191 – 192.
- Rimler, R., Sandhu, T. and Glisson, J. (1998): Pasteurellosis, Infectious serositis, and Pseudo tuberculosis. In: A laboratory manual for the isolation and identification of avian pathogen, 4<sup>th</sup> 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/leaf lets](http://www.Spc.int/rahs/publication/leaflets), accessed on June 12, 2010.

- Shewan, P. (1986): *Pasteurella*. In: Gyles, C. and Thoem, C., Eds., Pathogenesis of Bacterial Infection in Animals. Iowa State University press, Pp 147 - 153.
- Sonaiya, E. (1990): The context and prospects for development of small holder rural poultry production in Africa. CTA - Seminar proceedings on small holder rural poultry production held in Thessaloniki, Greece, 9 - 13 October 1990. Pp 135 - 152.
- Tadelle, D., Million, T., Alemu, Y. and Peters, K. (2003): Village chicken production system in Ethiopia. Use patterns and performance evaluation and chicken products and socio economic functions of chicken. Debre Zeit Agricultural Research center, Debre Zeit, Ethiopia. *Live st. Res Rural. Dev.* **15**. [http:// www.Cipavorg. co/1rrd/1rrd 15/1/ tadeb 151.htm](http://www.Cipavorg.co/1rrd/1rrd15/1/tadeb151.htm), accessed on June 10, 2010.
- Tadesse, S., Ashenafi, H. and Aschalew, Z. (2005): Sero prevalence study of Newcastle disease in local chickens in central Ethiopia. *Int. J. Appl. Res. Vet. Med.*, **3**:25-29.
- Thrusfield, M. (2005): Veterinary epidemiology. 3<sup>rd</sup>ed. Black Well Science Ltd. UK, Edinburgh. Pp 228 - 246.
- Vogel, J. and Parrot, C. (1994): Mortality survey in feed yards, the incidence of death from digestive, respiratory and other causes in feed yards on the Great Plains. *Comp. Cont. Ed. Prac. Vet.*, Pp 227 - 234.
- Whiteman, C. and Bickford, A. (1989): Avian disease manual, American association of avian pathologists, 3<sup>rd</sup> ed. Kendall/Hunt Publishing Company, Iowa, USA. Pp 98 - 102.
- Yates, W., Jericho, K. and Doige, C. (1983): Effect of viral dose on experimental pneumonia cause by aerosol exposure of fowls to respiratory virus. *Can. J. Comp. Med.*, **47**: 57 - 63.
- Yong - ku, W. and Kim J. (2006): Fowl cholera outbreaks in domestic Poultry and Epidemiological properties of *P. multocida* isolates. *Micro Biol. J.*, **15**: 344 - 353.

Zelege, A., Gelaye, E., Sori, T., Ayelet, G., Sirak, A. and Zekarias, B. (2005): Investigation on infectious bursal disease outbreak in Debre Zeit. Asian net work for scientific information. *Int. J. poul. sci.*, **7**: 504 - 506.

Zeng, H., Panthers, G. and Murry, M. (1999): Molecular cloning of the *P. haemolytica* Pom a surface domain. *J. Infect. Immunol.*, **67**: 4968- 4973.

8. ANNEXES

Annex 1: Sample collection format for bacteriology

| S/n | date | farm | breed | age | health status | sample taken | Method of preservation | Sample code |
|-----|------|------|-------|-----|---------------|--------------|------------------------|-------------|
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |
|     |      |      |       |     |               |              |                        |             |

**Annex 2:** Test results recording format for identification of bacterial isolates up to Genus level (primary identification).

| Sample Code | Gram reaction | Cellular Morphology | Growth on MacConkey agar | Catalase test | Oxidase test | Motility test | OF test | Name of the genus |
|-------------|---------------|---------------------|--------------------------|---------------|--------------|---------------|---------|-------------------|
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |
|             |               |                     |                          |               |              |               |         |                   |

**Annex 3:** Biochemical tests used for secondary identification of isolated bacteria

| Sample code | Indole Production | Urease | Nitrate reduction | Ornithine decarboxylase | Acid and gas production with in 24-48 hrs |      |      |      |       |
|-------------|-------------------|--------|-------------------|-------------------------|-------------------------------------------|------|------|------|-------|
|             |                   |        |                   |                         | Glu.                                      | Lac. | Suc. | Mal. | Mann. |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |
|             |                   |        |                   |                         |                                           |      |      |      |       |

**Annex 4:** Biochemical tests applied for differentiation of *P. multocida* subspecies

| Sample<br>Code | Fermentation of |          |             |          |          | Subspecies<br>differentiated |
|----------------|-----------------|----------|-------------|----------|----------|------------------------------|
|                | Trehalose       | D-xylose | L-arabinose | Sorbitol | Dulcitol |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |
|                |                 |          |             |          |          |                              |

## Annex 5: Serological test results recording format

[illegible]

### 1. Grams stain (Carter, 1984)

#### *Procedure:*

- A thin smear or film was prepared
- The film allowed to dry in air
- The film was fixed by passing through the Bunsen flame several times
- The slide was flooded with crystal violet for 30 to 60 seconds
- The stain was poured and the remaining stain was washed with iodine solution
- The iodine solution was washed off
- Decolorized with 95% alcohol
- Counter stained with safranin for 30 to 60 seconds and washed with water
- The slide was blotted with filter paper and examined under microscope using oil immersion objective

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

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

*Procedure:* A loopful of the bacterial growth was taken from the top of colonies, avoiding the blood agar medium. The bacterial cells were placed on a clean microscope slide and a drop of 3% H<sub>2</sub>O<sub>2</sub> was added. An effervescence of oxygen gas, within a few seconds, indicated a positive reaction.

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

*Principle:* The cytochrome oxidase enzyme is able to oxidize the substrate tetra methyl-p-phenylene diamine dihydro chloride, forming a colored end product Indophenol.

*Procedure:* A 1% aqueous solution of tetra methyl-p-phenylene diamine dihydrochloride was prepared. Then a piece of filter paper was moistened in a Petri dish with fresh reagent (tetra methyl -p- phenylene diamine dihydro chloride) and the test bacterium was streaked firmly across the filter paper with a glass rod. A dark purple color along the streak line within 10 seconds indicates a positive reaction.

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

*Principle:* The test is used to determine the oxidative or fermentative metabolism of a carbohydrate by the bacterium.

*Procedure:* O - F base medium was prepared and sterilized by autoclave at 121°C for 15 minutes. The O - F base medium was cooled to 50°C and 10% sterile glucose, sucrose and dextrose were added to basal medium by making the final concentration of each sugar 1% and dispensed into sterilized tubes. Two tubes of the O-F medium with sterile sugars were heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen and the tubes were then cooled rapidly under cold running water. Both tubes were stab inoculated with the bacterium and a layer of sterile paraffin oil was layered on top of one of the tubes (sealed tube) to a depth of about 1cm and the tubes were incubated at 37°C and examined in 24 hours and then daily for up to 14 days.

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

*Procedure:* SIM medium (BBL) was used to detect motility. The medium was sterilized by autoclave at 121 °C for 15 minutes. It was stab inoculated using a straight wire. Then the tube was examined for motility after incubation at 37°C for 14 and 48 hours. If there is a diffuse growth through out the medium, the bacterium is considered as motile. The growth of non motile bacteria is confined to stab line. To interpret the results, the tube was held against a good light and the inoculated tubes were compared with uninoculated one.

## Annex 7: Secondary identification

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

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

*Procedure:* SIM medium was stab inoculated with test bacterium and incubated at 37°C for 18 to 24 hours. Then Kovac's reagent (0.2ml) was added to tube and left for ten minutes.

*Interpretation:* The formation of dark red ring indicated positive test result while negative test result was indicated by the formation of a yellow ring.

### 2. Urease test (Quinn *et al.*, 2002)

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

*Procedure:* Appropriate amount of urea agar base was dissolved in sterile water and sterilized in autoclave at 115°C for 15 minutes and cooled down to 45 - 50°C in water bath. 40% sterile urea was added and mixed well. The medium was dispensed into sterile tubes and inclined to form slant. The surface of the agar slant was streaked with the test organism and incubated at 37°C for 18 to 24 hours.

*Interpretation:* Organisms that hydrolyze urea rapidly may produce positive reaction within 1 or 2 hours. Red (pink) color throughout medium indicated positive reaction.

### 3. Nitrate reduction test (Quinn *et al.*, 2002)

*Principle:* Nitrate reducing bacteria possess the enzyme nitrate reductase which reduces nitrate to nitrite.

*Procedure:* The pure colony to be tested was inoculated into nitrate broth (sterilized by autoclave), and cooled down in water bath. The inoculated test tubes were incubated at 37°C for 12 - 24 hours. Then 5 drops of each reagent "A" and "B" (A= 0.5 %  $\alpha$ -naphthylamine in 5N acetic acid, B= 0.8% sulphanilic acid in 5N acetic acid) was added.

*Interpretation:* The formation of red color after addition of the reagent A and B indicated positive reaction, while there was no color change in negative reaction.

#### 4. Ornithine decarboxylase test (Quinn *et al.*, 2002)

*Principle:* Decarboxylase is an enzyme possessed by many species of microorganisms that can decarboxylate amino acid ornithine to putrescine.

*Procedure:* The pure colony of organism to be tested is inoculated into 0.5% L-ornithine dicarboxylase broth with and without amino acid ornithine and incubated at 37°C up to 4 days.

*Interpretation:* The formation of yellow color from purple in test tubes with and without ornithine showed fermentative action of the organism to glucose, while only a tube with ornithine changed back to purple showed positive reaction.

#### 5. Carbohydrate fermentation tests (Quinn *et al.*, 2002)

*Principle:* Many microorganisms possess enzymes which can ferment sugars and alcohols by producing acid with and without gas.

*Procedure:* The pure colony of organism to be tested was inoculated into phenol red broth with sterile 1% or 0.5% (salicin) of final sugar concentration and Inverted Durham tubes were put. The bottles were incubated for 24 - 48 hours at 37°C.

*Interpretation:* The change of the color of phenol red broth from red to yellow without the formation free space on the top of inverted Durham tube indicated the bacterium can ferment the sugar without gas production; if the color of the broth was changed from red to yellow with formation free space on the top of inverted Durham tube, the bacterium can ferment sugar with production of acid and gas. But if there was no color change, either the bacterium did not ferment a given sugar or did not grow on basal medium.

## **Annex 8: Media used for isolation and identification of *P multocida***

### **1 Tryptose soya broth (Oxoid, Hampshire, England)**

*Preparation:* 30.0gm of medium was dissolved in one liter of distilled water and distributed into final containers, sterilized by autoclaving at 121°C for 15 minutes.

### **2. Tryptic soya agar (Difco, Sparks, USA)**

*Preparation:* Forty grams of the powder was suspended in one liter of distilled water and mixed thoroughly. The medium was heated with frequent agitation and boiled for one minute to dissolve completely. Then autoclaved at 121 °C for 15 minutes cooled to 45 - 50°C at water bath and dispensed to sterilized Petri dishes or test tubes.

### **3. Blood agar base (Titan Biotech Limited, Bhiwede )**

*Preparation:* Forty grams of the powder was dissolved in a liter of distilled water by heating. Sterilized by autoclaving for 15 minutes at 121°C. The base was cooled to 45°C to 50°C and 50 - 70 ml defibrinated blood was added in a liter of base and mixed properly and poured to sterile Petri dishes.

### **4. MacConkey agar (Oxoid, Hampshire, England)**

*Preparation:* 51.5gms was suspended in 1 liter of distilled water, and brought to boil completely. Sterilized by autoclaving at 121 °C for 15 minutes.

### **5. SIM medium (BBL, Becton Dickinson, USA)**

*Preparation:* 30gms of medium was suspended in one liter of distilled water, mixed thoroughly, Heated with frequent agitation and boiled for one minute, and autoclaved at 121°C for 15 minutes.

### **6. O F basal medium (Merck, Darmstadt, Germany)**

*Preparation:* 11gms of medium was suspended in one liter of distilled water by heating, autoclaved at 121°C for 15 minutes: at approximately 50°C 900 ml of basal medium was mixed with 10% of D (+) glucose, sucrose and dextrose. Dispensed in sterile test tubes.

### **7. Urea Agar Base (BBL,® Becton Dickinson, USA)**

*Preparation:* 40gms of urea powder was suspended in 1000ml of distilled water, mixed thoroughly and sterilized by filtration. 15gms of urea agar base was suspended in 900ml distilled water, autoclaved at 115°C for 15 minutes, cooled to 50°C and 100 ml of filtered

urea was added. It was mixed thoroughly and dispensed aseptically in sterile tubes, cooled in slanted position so that deep butts were formed.

#### 8. Ornithine decarboxylase broth (Merck, Germany)

*Preparation:* 9gms of base medium was dissolved together with 5gms of ornithine in one liter of de mineralized water. Base medium was prepared with out ornithine as control. Filled into tubes up to about 3 cm: autoclaved at 121 °C for 15 minutes.

#### 9. Phenol-red broth base (Merck, Germany)

*Preparation:* 15 gm of medium was suspended in 1 liter of distilled water, mixed thoroughly and sterilized by autoclave at 121°C for 15 minutes, cooled down in water bath, at 45 - 50°C and the test sugars were added by making their final concentration 1.0% or 0.5%(salicin).

## Annex 9: Reagent

### 1. Phosphate buffered saline solution (PBSS)

|                                    |        |
|------------------------------------|--------|
| Nacl                               | 8.0gm  |
| Kcl                                | 0.2gm  |
| NaHPO <sub>4</sub> 2H <sub>2</sub> | 1.13gm |
| CaCO <sub>3</sub>                  | 10.1gm |
| Distilled water                    | 1000ml |

Adjust PH to 7.4 and autoclaved at 121 °C for 15 minutes

### 2. Alsever's solution

|                 |         |
|-----------------|---------|
| Dextrose        | 18.6gm  |
| Nacl            | 4.18 gm |
| Sodium Citrate  | 8.0gm   |
| Distilled water | 1000ml  |

Sterilized by steaming

Farm code

1-----Alema farm

2-----Genesis farm

## 9. DECLARATION

This thesis is my original work, has not been presented for degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name: Dereje Baye Wubie

Signature



Date of submission

28/10/2002 E.C

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

**Academic Advisors**

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