PATHOLOGICAL AND MOLECULAR CHARACTERIZATION ON CLINICALLY SICK CHICKENS WITH INFECTIOUS BRONCHITIS VIRUS IN SMALL SCALE AND COMMERCIAL POULTRY FARMS IN BISHOFTU TOWN, ETHIOPIA

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MVSC IN VETERINARY PATHOLOGY

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A Thesis Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in the Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Pathology

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APPROVAL

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As a member of the Board of Examiners of the MVSc Thesis open defense, we certify that we have read and evaluated the Thesis prepared by Hana Desta Demeke entitled **"Pathological and Molecular Characterization On Clinically Sick Chickens With Infectious Bronchitis Virus In Small Scale and Commercial Poultry Farms In Bishoftu Town, Ethiopia''** and examined the candidate. We recommend that the Thesis be accepted as fulfilling the Thesis requirements for the degree of Master of Science in Veterinary Pathology.

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DEDICATION

I dedicate this thesis manuscript to my sweet daddy, Desta Demeke Tesfaye and to my beloved husband, Dr Nafyad Worku Bedhane. My dad is a special father in the world because he caring me both as my father and mother until now. He sacrifices his all things that he has for me. I hope this work will inspire them to great heights.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my own work and recognition has been given for the sources of all material used in the preparation of this thesis through citation. This thesis has been submitted in partial fulfillment of the requirements for Master of Science degree at Addis Ababa University, College of Veterinary Medicine and Agriculture. The thesis is kept at the library of the University in order to be accessible to loaners under the rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBRIVIATIONS

%	Percent
AHI	Animal Health Institute
C-DNA	Copy-Deoxyribonucleic Acid
H&E	Haematoxylin and Eosin
IB	Infectious Bronchitis
IBV	Infectious Bronchitis Virus
ILT	Infectious laryngotracheitis
km	Kilometer
ND	Newcastle Disease
Rpm	Revolution per minute
RNA	Ribonucleic Acid
rRT-PCR	Real-time Reverse Transcription PC
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
VTM	Virus Transporting Media

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ABSTRACT

Poultry diseases are considered to be one of the most leading factors which are responsible for reducing both the total number and productivity of chickens. Infectious bronchitis virus is internationally important disease of high concern due its adverse effects on poultry production in different aspects. The Cross-sectional study was conducted from November, 2021 to June 2022 in Bishoftu town, Ethiopia with the aim of confirming the presence of infectious bronchitis virus (IBV) in the samples collected from suspected chicken through the combined result molecular, gross and histopathology. Depression with different severity, respiratory related signs, diarrhea and retardation in growth were the clinical signs which frequently seen and recorded in majority of them. Tissue (lungs, trachea and kidneys) and tracheal swab samples were collected from 40 chickens clinically suspected of infectious bronchitis virus. Among these 25 were from farm 1 (Alema) and 10 were from farm 2 (Elfora) commercial poultry farms and 5 were from farm 3 (Ato Yared small scale farm. The texture, shape, size and color of the organs were properly examined for gross pathology characterization. Accordingly, mild to severe tracheal hemorrhage, unevenly distributed color on the kidney and lung were observed during gross examination of the organs. From all grossly examined organs, the most frequently affected organs were the trachea, 22(55%) and kidney, 15 (37.5 %) followed by lung, 11 (27.5 %). From 40 swab specimens collected for molecular characterization, 8 were found positive for IBV. From a total of 32 tissue samples, 20 were found positive by histopathological analysis and the microscopic lesions described were infiltration of inflammatory cells at different condensation state, mild to severe congestion and hemorrhages, tracheal mucosal epithelium damage, degenerative changes in the renal tubule. The overall results of the current study indicate the production and reproductive performance of chickens in the study area were highly influenced by the presence of infectious bronchitis virus. Thus, further scientific investigation should be conducted through using advanced diagnostic tools for explaining the disease in vast.

Key Words: *Chickens, Ethiopia, Gross lesion, Infectious bronchitis virus, Histopathology, PCR.*

1. INTRODUCTION

In Ethiopia, poultry production has a tiny role to the income of producers as well as the country as compared to its number (Mamo *et al.*, 2013). Poultry production system can be grouped in to village or backyard, small scale and commercial (Fitsum and Aliy, 2014). Rearing of chickens in village production system is the most common practice in Ethiopia (Chaka *et al.*, 2012). Moreover, approximately 99% of total poultry population is reared under this system (Geleta *et al.*, 2013). Thus, because of the occurrence of low biosecurity measures and great risk of infectious diseases in backyard poultry production system, poultry diseases are considered to be the most important factor for reducing both the total number and productivity of chickens (Geresu *et al.*, 2016). Indeed in backyard production system chickens are able to move freely around their environment so they could be potential reservoir of infection to themselves as well as the commercial poultry farms (Emikpe *et al.*, 2010). From these infections, viral diseases are the top economic risk to poultry farm. Among viral diseases of chicken, newcastle disease (ND) and infectious bronchitis (IB) are the most commonly occurred respiratory disease within the chicken farms (Barberis *et al.*, 2018).

Infectious bronchitis was first recognized in the North Dakota of United States in 1931 by Schalk and Hawn. Initially, the disease was considered as to occur primarily in young chickens (Bande *et al.*, 2016) and to cause only respiratory disease but through time as some studies reported chickens of all age were susceptible (Awad *et al.*, 2014) although its severity is higher in young chicken than adult (Bande *et al.*, 2016) and the virus has been isolated from various body tissue such as respiratory, urinary and reproductive tract (Awad *et al.*, 2014). IB is one of the most widely distributed and highly contagious diseases of chicken caused by a single stranded RNA genome virus (Alsultan *et al.*, 2019), known as infectious bronchitis virus (IBV; *Avian coronavirus*) (Khataby *et al.*, 2016), which belongs to the order *Nidovirales*, family *Coronaviridae*, and genus *Gammacoronavirus.* It is a major cause of significant economic losses in the poultry industry by declining their production in terms of quality and quantity, mortality, poor weight gain, demand of extra costs for treating and controlling the disease (Barberis *et al.*, 2018).

Spreading of IBV between chickens and farms occur rapidly through inhalation of respiratory droplets and mechanical means. There were no any studies which indicate the vertical transmission of the virus (Ramakrishnan and Kappala, 2019). IBV is predominantly considered as a respiratory pathogen because once it enters to the host, primarily it targets the upper respiratory tract especially the epithelium of chicken trachea and mucus secreting glands to replicate themselves (Khataby *et al.,* 2016). It can causes ciliated epithelial cells damage in the trachea and other pathological alterations. However, The virus can also spread to other internal organs from initial replication site, such as kidney, oviduct and digestive tracts (Ramakrishnan and Kappala, 2019).

The age, sex, type and immune status of the host, the strain, virulence and dosage of the virus, involvement of secondary pathogens and the environment have great role in the severity and occurrence of the disease (Awad *et al.,* 2014). Generally the disease is characterized by coughing, sneezing, difficulty in breathing, nasal discharge, tracheal rales, decrease of feed intake, reluctant to move, dullness, lethargy and reduced weight gain in broiler (Kanwal *et al.*, 2018). Further it can causes a syndrome known as "false layer syndrome" in layers (Bande *et al.*, 2017) and some clinical signs might be observed as a result of the effects of IBV in the reproductive tracts of the chicken such as decrease in egg production, egg contain watery albumin, formation of thin, wrinkled and easily breakable egg shells (Balestrin *et al.*, 2014)..

Since clinical signs of most poultry diseases are not specific, advanced diagnostic tools are needed for their correct identification (Abed, 2014). Diagnosis of IBV infections can be made either by detection of IBV virus itself or the specific antibody response. Control and prevention of IBV caused infection is still becoming difficult even by using currently available vaccines because of the lack of cross-protection between different strains of IBV and the emergence of new IBV variant through spontaneous mutation and genetic recombination (Awad *et al.*, 2014). The seroprevalence of infectious bronchitis virus and identification of infectious bronchitis virus serotype in Ethiopia was indicated by the research of Tesfaye *et al.,* (2019), Tegegne *et al.*, (2020), Roba *et al.* (2021) and Shiferaw *et al.* (2022). However, till today there is scarcity of documented information about pathological findings on the organs of IBV suspected chicken inaddition to molecular characterization. Therefore, the main objectives of the current study were

to identify the pathological changes induced by infectious bronchitis virus on the vital organs of clinically sick chicken.

2. LITERATURE REVIEW

2.1. History of Infectious bronchitis virus

New disease of chicken with respiratory symptom was recognized at North Dakota of United States in 1931 by Schalk and Hawn. The disease is characterized by having respiratory signs such as gasping (Ramakrishnan and Kappala, 2019). Earlier, it was believed to occur primarily in young chickens (Bande *et al.*, 2016) and to cause only respiratory disease. However, later it was occurred in chickens of all age and isolated from various non-respiratory tissues like kidneys, reproductive and alimentary tracts (Awad *et al.*, 2014). At the time of its recognition, the nature of the causative agent was not determined. In 1933, a disease that has similar clinical symptom was reported by Bushnell and Brandly (Ramakrishnan and Kappala, 2019) and they were considered that the causative agent was a filterable virus since the disease transmitted by Berkefeld filtered material (Fabricant, 1998). They indicated that the disease was infectious laryngotracheitis (ILT). However, important tests were not made so the identity of the disease was not confirmed. In 1936, Beach and Schalm were found the causative agents of the disease as it was a virus which named as IBV (Abro, 2013) and made further critical testes known as cross-immunity studies in chickens to clearly differentiate ILTV from IBV (Ramakrishnan and Kappala, 2019). After the first IBV serotype was identified, the virus was reported in different parts of united states of America and a wide range of different IBV serotypes and genotypes were described worldwide in addition to the initially identified Massachusetts (MA) type of IBV (Abro, 2013). In Africa, the virus was recognized and reported for the first time in 1954 specifically in Egypt by Ahmed from birds with respiratory problems (Tegegne *et al.*, 2020) and ten years later, it was confirmed by Eissa et al. (1963). The virus was recognized in Morocco in 1983 (El Houadfi and Jones, 1985). As the report of Tesfaye *et al.*, (2019) four serotypes of infectious bronchitis virus were identified from unvaccinated backyard and commercial farms in Ethiopia namely M41, D-274, 793B and Qx.

2.2. Etiological Agent

There are four genera belongs to coronavirus namely *alpha, beta, gamma* and *delta* (Zhao *et al.,* 2014). Coronaviruses are very host specific; *alpha-* and *betacoronaviruses* only infect mammals, *gammacoronaviruses* only infect poultry, and *deltacoronaviruses* have a wider host range and include wild birds and some mammals. The name corona is from Latin which means crown since it has distinctive club shaped spike structures on the surface (Aziz, 2020). IB is a highly contagious viral disease of chickens (Ayim-akonor *et al.,* 2018). Similar to other coronaviruses, avian infectious bronchitis virus genome consists of a linear, non-segmented, positive-sense, and single-stranded enveloped RNA (Rafique *et al.,* 2018). The virus is classified under the genus Gammacoronavirus, family Coronaviridae, and order Nidovirales (Alsultan *et al.,* 2019). The genome of the virus is approximately 27 kilo-bases (kb) in length with club-shaped spikes in their surface. The virus contains four main structural proteins; spike glycoprotein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N) (Han *et al.,* 2020). The S protein is located on the surface of the virus external membrane **(figure 1)** so it is cleaved into S1 and S2 glycoproteins during viral binding to the host cell (Moharam *et al.,* 2020).

The S1 subunit of S gene contains epitopes for virus neutralization, cell attachment, and serotype specificity (Rohaim *et al.,* 2019). Therefore, S1 shows high sequence variability than S2 subunit. The main responsibility of the S2 subunit of S protein is supporting the S1 protein to the viral membrane (Feng *et al.,* 2014). Both M and E proteins are membrane-associated proteins required for the formation of virus-like particles and virus budding. N protein is associated with the RNA genome and forms the ribonucleoprotein (Xu *et al.,* 2016). Because of the existence of hypervariable regions (HVRs) on spike protein which is responsible for its variation, it is widely studied rather than the other structural proteins (Tegegne *et al.,* 2020). The emergence of new serotypes of IBV is as a result of gene mutations in S1subunit (Khataby *et al.,* 2016). The virus is fragile because it is easily destroyed by disinfectants, sunlight, heat and other environmental factors (Butcher *et al.,* 2022).

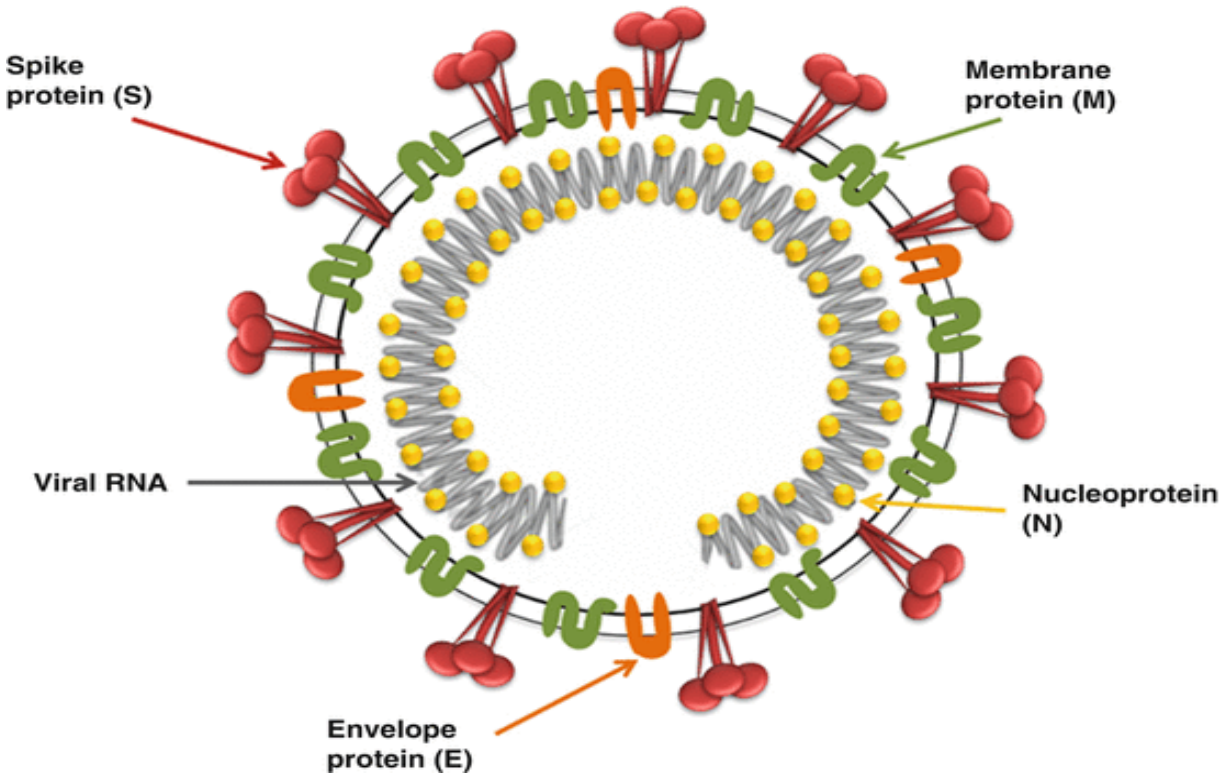


Figure 1: Schematic diagram of infectious bronchitis virus. (**Source:** Abdel-Moneim, 2017)

2.3. Mode of Transmission

Infectious bronchitis virus is an acute and highly contagious viral disease of chickens (Lian *et al.,* 2021). The virus has relatively short incubation period (18 - 36 hours). Viral transmission can occur horizontally by means of aerosol and mechanical (Abozeid and Naguib, 2020). Aerosol transmission is a significant mode of transmission during the first 3–5 days of post infection because high concentration of the virus is present at this time in the respiratory tract. Nevertheless, its concentration drops rapidly even below the level of detection within the second-week of post infection (Rana *et al.,* 2021). There was no any documented information which indicates the vertical transmission of the virus but its presence on the surface of the egg shell is evident (Ramakrishnan and Kappala, 2019). Among farms transmission is usually associated with contaminated people, equipment and vehicles (Dhama *et al.,* 1014). Recent data also indicates that the virus has the ability for venereal transmission (Manswr, 2018).

There are some strains of IBV that have capability of multiplying in the tissues of the alimentary tracts but usually causing of significant pathological change on the digestive tissues are rare (Awad *et al.,* 2014). However, there may be a continuous releasing of the virus through faeces to the surrounding environment and this can contaminate feed, water, chicken attendant and vehicles. Wild birds are the major sources of indirect transmission to different regions (Abro, 2013).

2.4. Pathogenesis

IBV is an epitheliotropic virus that attacks organs lined with epithelia such as respiratory tract, urogenital and alimentary tract (Houta *et al.,* 2021). Upper respiratory tract is the initial target organs of IBV to multiply mainly in the tracheal epithelium and mucus secreting glands (Villarreal *et al.,* 2010). These can induce various mucosal pathological changes such as loss of cilia, degeneration and necrosis of epithelial cells, glandular degeneration, inflammatory cell infiltration and epithelial hyperplasia (Okino *et al.,* 2017). The pathogenesis of the disease differs according to the system involved as well as the strain of the virus. Severe disease can occur mainly due to the predisposition of chickens to secondary bacterial infections such as colibacillosis and mycoplasma infection after tracheal ciliary losses (Houta *et al.,* 2021).

Depending on the virus strain and the immune status of the host, the virus causes viraemia and disseminated to other non-respiratory epithelial tissues where secondary replication can occur like kidneys, testes, oviduct and gastrointestinal tract (Abro, 2013). The kidneys are the main replication site for nephropathogenic IBV strains after their initial replication in the respiratory tract. The virus may then resulting in damaging of ciliated cells of the nephron, including proximal, distal and collecting tubules (Houta *et al.,* 2021). The kidneys become pale, discolored and enlarged following infection with nephropathogenic IBV. Urate deposits are also commonly observed with tubular distention. Histologically, recruitment of mononuclear cell in interstitial tissues and degenerative changes are seen in the tubules (Reddy *et al.,* 2016).

2.5. Clinical Signs

Once the birds infected with IBV, they usually develop clinical signs within short period of time mainly 24-48 hours. Even if all age group of chicken are susceptible to IBV, susceptibility and mortality rate is higher in young chicken than adult. Mortality rate usually ranges 20-30% and even it may reach up to 80% depending on the infecting IBV strain virulence and other related factors (Awad *et al.,* 2014). In adult chicken the disease as well as the morbidity and mortality rate may not be serious as occur in young chickens but if IBV is mixed with other pathogens like secondary bacterial infection more severe diseases and increased mortality rate can occur in all age group (Alsultan *et al.,* 2019). The time of infection determines the extent of the damage. The general signs of the disease is characterized by coughing, sneezing, difficulty in breathing, nasal discharge, tracheal rales, anorexia, reduced body weight gain, depression, lethargy, growth retardation in broilers (Najimudeen *et al.,* 2020). Although IB is mostly known as a respiratory disease and causes respiratory related clinical signs more frequently, nephropathogenic strains initiate severe damage to the kidney and produce significant signs associated with kidney problem and lesions rather than respiratory signs and lesions (Awad *et al.*, 2014). Besides its general signs, nephropathogenic IBV strains result in weight loss, watery droppings, increased water consumption and an increase in the incidence of mortality (Najimudeen *et al.,* 2020).

In affected layer chicken, the following typical clinical signs may be observed such as drop in egg production, change in external and internal egg's quality and false layer syndrome (Alsultan *et al.,* 2019). False layers syndrome can be induced when layer chicken exposed to the virus at its early age, then the virus infects the oviduct and alter its normal development (Gallardo, 2021). In some cases, permanent loss of egg production may be seen when severe damage to the reproductive tract is occur (Rahim *et al.,* 2018). Although IBV infection also occurs in reproductive tracts of male chickens, little attention has been given to its consequence. According to recent investigations, there are IBV strain that targets the testes and causes low sperm production and infertility. IBV replication in the cells of seminiferous tubule was indicated by the research of Gallardo *et al.* (2011). Moreover, they also demonstrated transmission of IBV via infected semen (Reddy *et al.,* 2016).

Even if some IBV strains have ability to replicate in tissues of the alimentary tract like oesophagus, proventriculus, part of small intestine, caecal tonsils, rectum, and cloaca, significant pathological change may not be observed (Awad *et al.,* 2014) but the virus presents for long periods within the digestive system and excrete through faeces (Sun *et al.*, 2011).

2.6. Diagnosis

Infectious bronchitis can be diagnosed on the basis of clinical manifestation of the disease, detection of the induced antibody against IBV, antigen or viral DNA in the tissue sections and clinical materials (Dhama *et al.,* 1014). The type of sample, availability of test materials and facilities, test reporting time, test purpose, and the place where the test is carried out determines the choice of the test (Bande *et al.,* 2016).

2.6.1. Clinical Signs

The host (age, sex, type and immune status), the virus (strain, virulence and dosage), and involvement of secondary pathogens have great impact in the severity and occurrence of the disease clinical signs (Awad *et al.*, 2014). Diagnosis of a certain disease begins with evaluation of clinical signs and symptoms. Clinical signs like coughing, sneezing, dyspnea, nasal discharge, tracheal rales, loss of appetite, reduced body weight gain, depression, lethargy, growth retardation are suggestive of IBV (Hines and Miller, 2012). The clinical parameters and signs are useful as preliminary evidences during infectious bronchitis (IB) disease diagnosis. Nevertheless, signs induced by IBV are not pathognomonic (Legnardi *et al.*, 2020), use of other laboratory diagnostic methods is mandatory to differentiate IBV from other upper respiratory pathogens such as ILT , avian influenza and Newcastle disease (ND) (Manswr, 2018) as well as IBV strains characterization. Laboratory diagnostic techniques used for IBV include viral isolation, serological and molecular assays (Legnardi *et al.,* 2020).

2.6.2. Pathological Lesion

Since IBV affects primarily the upper respiratory tract of chicken, infection is mainly associated with damaging the mucosa of respiratory tract particularly the trachea and mucus secreting glands resulting in hemorrhagic lesions in the mucosa of the trachea (El-Fetouh *etal.*, 2016).
In IBV infected chicken, the respiratory tract may shows serious, catarrhal and caseous exudates in the nasal passage, sinuses and trachea. Air sac affections are characterized by thickening and opacity (Dhama *et al.*, 1014). The dissemination of virus to other non-respiratory epithelial tissues is determined by the tissue tropism characteristics of the invading virus (Rana *et al.*, 2021). For nephropathogenic IBV strains, the kidneys are the main replication site. Grossly, moderate to severely enlarged and pale kidneys are observed (Houta *et al.*, 2021). Pathology is not usually associated with infection of alimentary tracts by enterotropic IBV. Pathogenic strain causes thickened, hemorrhagic or ulcerative lesion in the proventriculus, hemorrhagic lesion in the caecal tonsils and thickening of duodenum (Dhama *et al.*, 1014). In addition to gross findings, histopathological findings also yield a variety of descriptive lesions. Microscopically, inflammatory cell infiltration in the interstitial tissues, renal tubular cells degeneration and necrosis may be seen in the kidneys (Okino *et al.*, 2017). Degenerative ovarian follicles, inflammatory cell infiltration and desquamation of epithelial cells in the oviducts were common in the infected flock (Hassan *et al.*, 2021). Generally, diagnosis should not be based on pathological lesions or clinical signs alone because those types of lesions and signs are not specific to any strain of IBV. Therefore, Pathology is a useful tool to guide disease diagnosis, but it cannot be used solely to diagnose IBV (Hines and Miller, 2012).

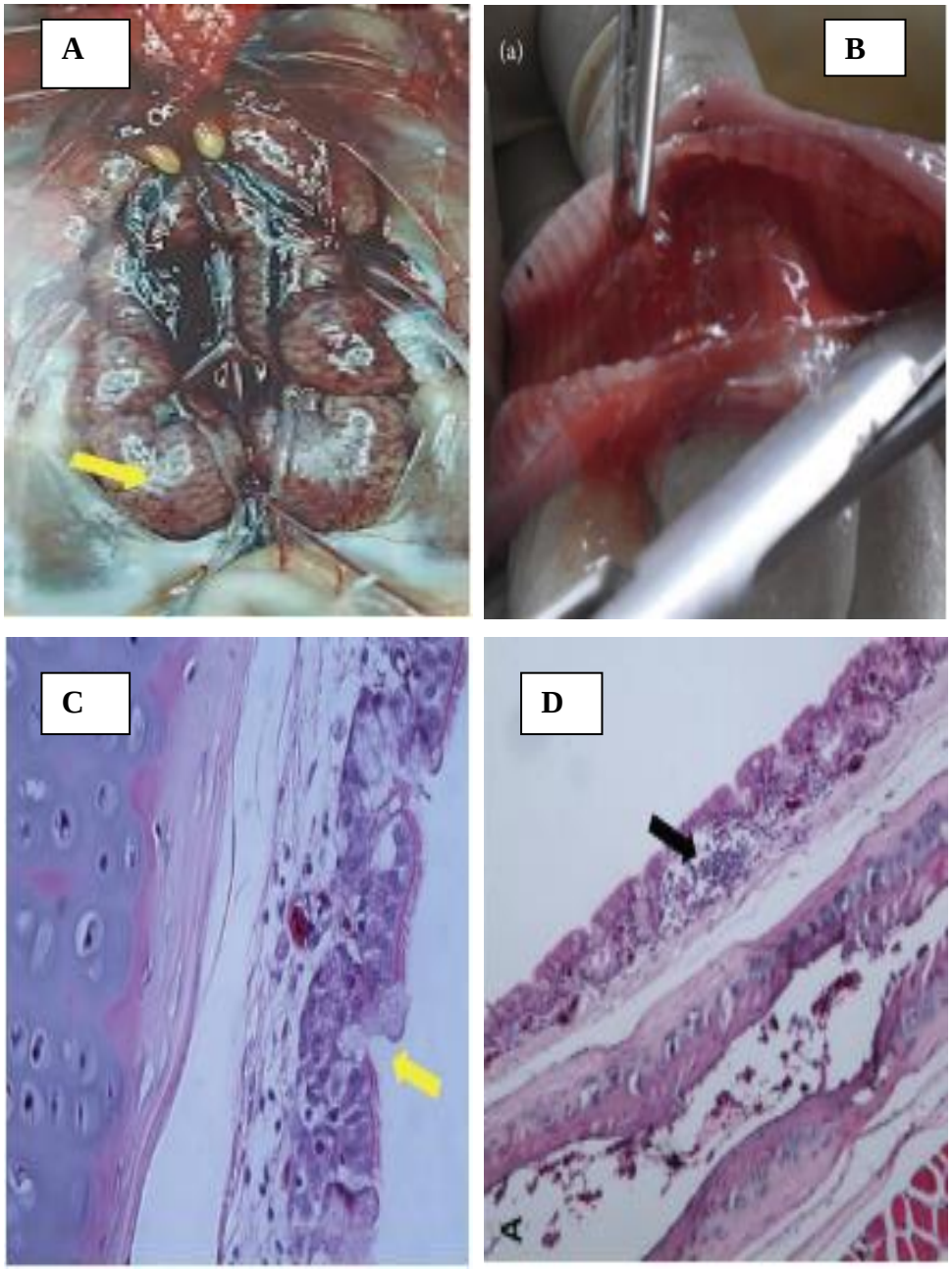


Figure 2: Histopathological changes in naturally IBV infected chicken.

Keys: (A) Swelling and congestion of the kidney; **(B)** Presence of mucoid secretion, congestion, and hyperaemia in the trachea**; (C)** Evidence of mucosal secretions of goblet cells, yellow arrow**; (D)** The marked infiltration of lymphocytes within the epithelia indicated by black arrow (Bande *et al.*, 2016)

2.6.3. Laboratory Techniques

I. Serological techniques

Serological methods are used to assess the induced immunological response against IBV. Agar gel precipitation (AGP), enzyme-linked immunosorbent assay (ELISA), virus neutralization (VN) and hemagglutination inhibition (HI) are different types of serological test (Butcher *et al*.,, 2022). Commercial ELISA tests are the most commonly applied serological tests beyond the others because of its cost-effectiveness and rapid response. The majority of the available ELISA kits are designed to detect polyclonal antibodies against the whole virion and do not allow serotypization (Legnardi *et al.*, 2020). Due to the emergence of different IBV serotypes and the absence of good cross-reaction between them serological tests are less applicable and nonconclusive in classifying new or emerging IBV isolates (Bande *et al.*, 2016).

II. Virus isolation

Indeed virus isolation is considered the gold standard for IBV diagnosis. However, it is time consuming, laborious and requires several passages in embryonated chicks (Manswr, 2018). To made successful IBV isolation, appropriate samples should be taken in the right time of the disease (Bande *et al.*, 2016). Tracheal tissues or swabs are the sample of choice in the acute phase, but kidneys or oviduct are also suitable sites, especially more than one weeks after the start of infection. Samples should be carefully stored on ice and rapidly sent to the laboratory to preserve virus viability (Legnardi *et al.*, 2020).

III. Molecular technique

Recently, molecular assays are the most used tools for IBV detection because of its good sensitivity and quick response time. Further, it helps to characterize the detected IBV strains +naddition to viral RNA detection (Legnardi *et al.*, 2020).

The study virus has RNA as their nucleic acid. Since RNA has a less stable structure than DNA so the viral RNA should be reverse transcribed to single stranded complementary DNA (cDNA). Reverse transcription polymerase chain reaction (RT-PCR) is used to detect RNA virus which is negative and single stranded (Sundgren, 2020). Real time RT-PCR (rRT-PCR) is more sensitive and specific than other molecular assays, because this assay uses specific primers and probe. inaddition, quantification of virus or its genetic material can be detected (Alp Onen and Ozgur, 2017). rRT-PCR was developed by Mullis and Faloona in 1987. The ability to identify and quantify a particular nucleic acid in a sample without any post PCR manipulations is tremendous. A good way to generate both qualitative and quantitative data is the use of rRT-PCR. The technique of real-time PCR allows for detection of nucleic acid amplification as the PCR progresses by measuring the increase in fluorescence using a real-time PCR machine. In this manner, the identity and amount of a target nucleic acid can be determined (Rafique, 2018).

2.7. Prevention and Control

Understanding about the factors that could play a great role in the emergence of infectious bronchitis variants is necessary to perform surveillance and establish prevention strategies. proper management is a key component for holding disease-free flock (Gallardo, 2021) which includes strict biosecurity, good hygiene and sanitation practice, one age-system of rearing, cleaning and disinfection between batches along with judicious vaccination (Dhama *et al.*, 1014). Farm density has a great role in viral transmission among farms. Only having proper management alone does not prevent the disease transmission (Legnardi *et al.*, 2020). Thus, further preventing measures should be undertaken like vaccination. Both live attenuated and killed vaccines are used to control IBV in commercial poultry farm. Since serotypes of IBV do not cross-protect each other, multivalent vaccine containing two or more antigenic types would be beneficial in providing broad protection (Ramakrishnan and Kappala, 2019). Nevertheless, the continuous genetic and antigenic changes of the circulating IBV caused the emergence of new IBV variants which result in significant economic losses to the poultry industry (Okino *et al.*, 2017) and discourage the effectiveness of immunization program (Fathy *et al.*, 2019).

2.8. Infectious Bronchitis Disease Status in Ethiopia

IBV is the most important viral diseases that affect the respiratory, urogenital and reproductive tissues of chicken. All chicken age groups are susceptible to the virus but young chicks are more susceptible as resistance increases with age (Tegegne *et al.*, 2020). It induces huge losses not only to the chicken industry but also to the economy of the country because poultry production has a significant role in the economy of Ethiopia (Shiferaw *et al.,* 2022). Poultry diseases mainly viral pathogens are estimated as the main cause for the presence of unsatisfactory poultry production in Ethiopia. Although IBV was recently recognized in Ethiopia at lower occurrence levels (Hutton *et al*.,2017; Tegegne *et al.*, 2020), but now it become one of the most frequently occurring viral disease like infectious bursal disease (IBD) and ND (Wayou *et al.,* 2021). However, apart from reporting the seroprevalence and significant impact of IBV on both commercial and backyard chicken farms, it is not studied well; the government and other responsible organizations give more attention for ND and other respiratory diseases rather than IBV (Birhan *et al.,* 2021). All-in-all-out system of rearing, good biosafety measures along with vaccination is the best option for prevention and control of IBV (Dhama *et al.,* 2014). Nevertheless, as a result of unavailability of IBV vaccines currently in Ethiopia IBV control is difficult. Due to the absence of good diagnostic coverage in Ethiopia, most poultry disease outbreaks are undiagnosed and dead chickens are simply discarded usually in more remote parts of the country (Chaka *et al.*, 2012). This may provide a suitable environment for viral spreading.

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted from November, 2021 to June, 2022 in Bishoftu, Oromia regional state, Ethiopia. Bishofitu is located at the distance of 45km southeast of Addis Ababa, the capital city of Ethiopia (Figure 3). The area is located at 9^0 N latitude and 40^0 E longitudes with an altitude of 1880 meters above sea level. The climatic condition of the area is predominantly temperate. The average annual rainfall is 866mm. In these areas the rainfall pattern is bimodal which includes a long rainy season extends from June to September and 84% of the rain is expected from this season and the remaining in the short rainy season extending from March to May. The dry season extends from October to February. The temperatures of the area are ranges from 14°C to 26°C. The humidity of the area is 66% in summer and 56% in winter. Mixed farming system is followed in the area (CSA, 2017; Abunna *et al*. (2018)).

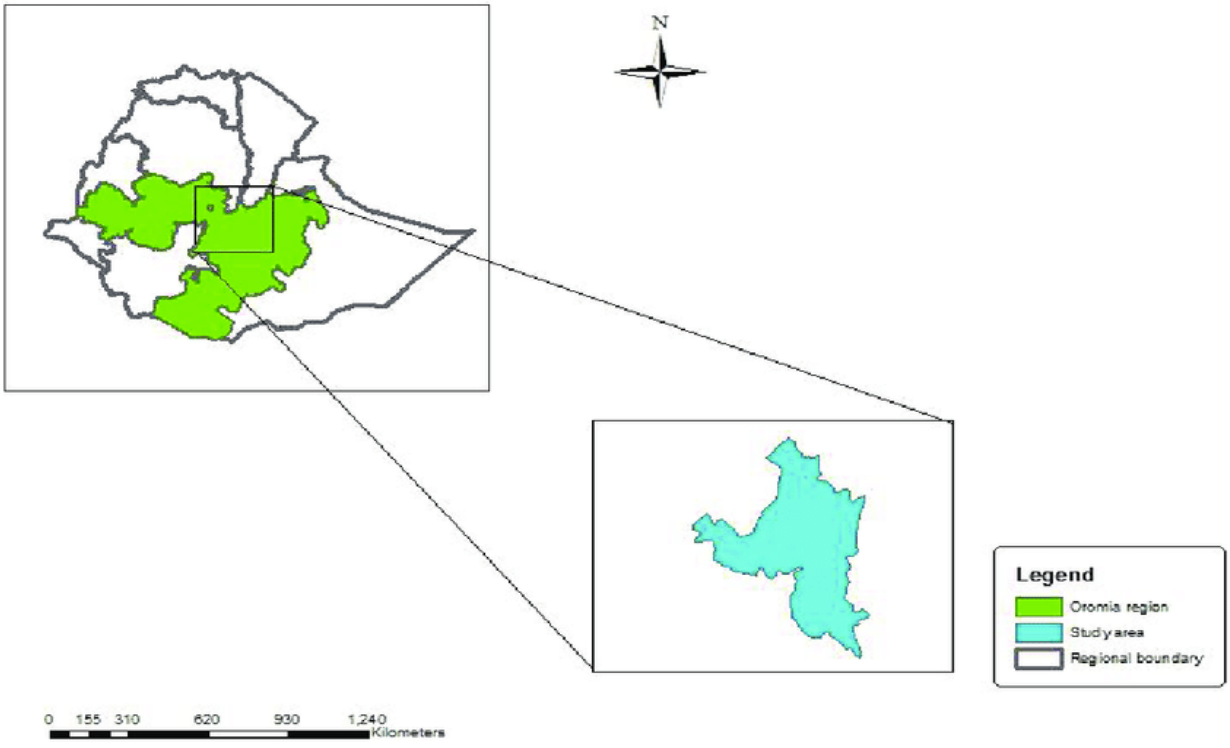


Figure 3: Schematic representation of study area

Sources: (Getachew *et al.*, 2020)

3.2. Study Population

The study was conducted on chickens which are clinically suspected with IB. Chickens were gathered from small scale and commercial poultry farms located in Bishoftu town, Oromia Regional State, Ethiopia. Chickens used in this study were from different farms (Alema, Elfora and Belay small scale farm) and all the necessary data were collected from the study population such as animal category (broilers or layers), breed, age, presence of clinical signs, production (intensive and extensive), housing system (backyard, commercial), population size, and vaccination (day of vaccination, number of vaccinations and vaccine strains used). Accordingly there was no vaccination history for the chickens against IBV. Overall, a total of 40 chickens were involved. Among these 25 were from Alema and 10 were from Elfora commercial poultry farms and 5 were from Ato Yared small scale farm. All of the chickens included for this study were from exotic breed and only broiler (Bovans Brown, Rose broiler and Cobb 500 broiler) that were kept under intensive management system.

3.3. Study Design and Sampling Methods

A cross-sectional study design with purposive sampling method was conducted to gather the necessary data from all study populations. Any IB suspected chickens from the selected small scale and commercial poultry farms were purposively sampled. Biopsy tracheal swab samples were taken from clinically suspected chickens by using virus transporting media (VTM) for molecular characterization and necropsy samples were taken for histopathology.

3.4. Sample Collections

Ante mortem examination was carried out on all study chickens. These included any abnormality in movement, reaction to touch and sound, any visible discharges from natural orifices, coughing, lesions on body and body conditions. The swab samples were collected from IB the trachea of IBV suspected chicken using VTM before euthanized the chicken and stored at -20^0c in refrigerator until processing.

Chickens were euthanizing through cervical dislocation and necropsy examination was performed according to the procedure described by (Butcher and Miles, 2018) **(annex 1).** Post mortem inspection was followed once ante mortem examination accomplished. The presence of any visible pathological change in various aspects of the organ such as its color, shape, size and texture were carefully examined and recorded. Any organ with gross pathological change was considered for histopathological examination. The representative pieces of tissue samples from the organs of all suspected chickens were collected by using bottle containing 10% neutral buffered formalin for histopathology examination. All collected samples were labeled with permanent marker in order to identify each collected samples easily.

3.5. Sample Transportation

The collected tracheal swabs were transported by using ice box to molecular laboratory of Animal Health Institute (AHI), Sebeta for molecular characterization and histopathological procedures were done in histopathology laboratory of AHI on the collected tissue samples using neutral buffered 10% formalin. The swabs were stored at -20°C refrigerator in the laboratory until processing.

3.6. Sample Processing

3.6.1. Histopathology

Small pieces of tissue samples approximately 5cm from the organ of clinically sick chickens were collected into a bottle that contains 10% neutral buffered formalin to preserve the tissue structure for histopathological examination. Tissue processing was done according to the procedures described by Chong et al. (2016) **(Annex 2).** The tissues were trimmed to approximately from 1 to 2 mm thickness. The procedure of tissue processing starts from dehydration so the trimmed tissues was dehydrated by keeping the tissue in increasing strength of ethyl alcohols (70, 95 and 100%) after fixating the sample adequately. Xylene was used as a clearing agent. The tissues were embedded with melted paraffin wax. All these procedures were done within the automatic tissue processing machine. Then the tissues were dislodges from

automatic tissue processing machine and paraffin was poured on the plastic tissue cassette to prepare tissue block. Once block was prepared, tissue was sectioned with a thickness of 5 micrometer by using microtome machine. The sectioned tissue was placed on the warm water which contained in water bath. Here the temperature of the water bath should be less than the melting point of the paraffin wax (56-58^{0}C). The floated tissues were adhered to microscopic slide by holding the microscopic slide at angle of 45 degree carefully. Precaution was taken when adhering the floated tissue because a small mistake in tissue adhering might have impact in the result. The tissue slides were passed through xylene for deparaffinization and rehydrated by passing through decreasing strength of ethyl alcohol from 100, 95 to 70%. Finally washed with running tap water and stained by routine haematoxylin and eosin stain (H and E). The tissue samples were again dehydrated by passing through ascending grades of ethyl alcohols (70, 95 and 100%) and cleared by xylene. Finally the stained slides were mounted by canada balsam and allowed to dry well, then examined under the light microscope starting from the lower (10x) to higher magnification (100x) power. Finally histopathological alterations were carefully described and recorded.

3.6.2. Molecular procedures

The collected samples were stored at −20°C until RNA extraction was performed. The viral RNA was extracted from the pooled tracheal swab samples using Viral Nucleic Acid Extraction Kit (Qiagen viral RNA mini kit, Germany) following the manufacturer's instruction **(Annex 3)**. Real time RT-PCR (rRT-PCR) is more sensitive and specific than other molecular assays because this assay uses specific primers and probe. inaddition, quantification of virus or its genetic material can be detected (Alp Onen and Ozgur, 2017). One-step rRT-PCR was performed by using amplification of target RNA according to the manufacturer's instructions (Roussan *et al.,* 2009). The extracted RNA samples were then subjected to one step-rRT-PCR to conduct reverse transcription (production of complementary DNA (cDNA)) and quantification simultaneously. The PCR was performed in a thermal cycler with an initial denaturation at 95°C for 15 min., followed by 42 cycles. denaturation for 30 seconds at 95°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C. Afterwards, a final extension for 10 min at 72°C was performed (Abd El Rahman *et al.*, 2015)

3.7. Ethical Consideration

The work of the current study was conducted after permitted ethical approval and statement given by the University of Addis Ababa, College of Veterinary Medicine and Agriculture, Bishoftu. The study animals were handled according to the World Organization for Animal Health (OIE) animal welfare guidelines during the study period. The current study was reviewed by Animal Research Ethics and Review committee of the University of Addis Ababa for its ethical soundness. The committee evaluated the importance of this study through different aspect then ethical clearance was obtained from the Ethics Committee of the College of Veterinary Medicine and Agriculture, Addis Ababa University (**Annex 4**).

3.8. Data Management and Analysis

All organized data was arranged, checked, coded and entered to Excel spread sheet (Microsoft® office excel 2007). R version 3.2 was used for descriptive analysis to describe the proportion of the result analyzed as frequency and percentages of the lesions in relation to the organs affected in all cases. In this way the types of the gross lesions recorded with the frequency and percentage within each case in every organ of the body was known and characterized accordingly.

4. RESULT

4.1. Clinical Signs

Careful recording of the history of chickens was essential for better examination before the commeasure of any detailed activities. The presence of any deviation from the normal behaviors of chicken was strictly checked either individually or in group. Thus, clinical examination was performed on 40 chickens for detecting any manifested abnormal clinical signs. According to the present study, various clinical signs were recorded such as sign related with respiratory problems (coughing, dyspnea, unilateral and bilateral nasal discharges), depression, diarrhea, the presence of thick and not easily detached dropping from the vent, anorexia, unwillingness to move and decreased body condition. Of these, the major clinical signs with their frequency and percentage were recorded as shown in the **Figure 3**. Among 40 chickens examined, depression and respiratory problems were the most frequent clinical signs that represented 72.5% (n= 29) and 62.5% (n=25) respectively as compared with other clinical manifestations.

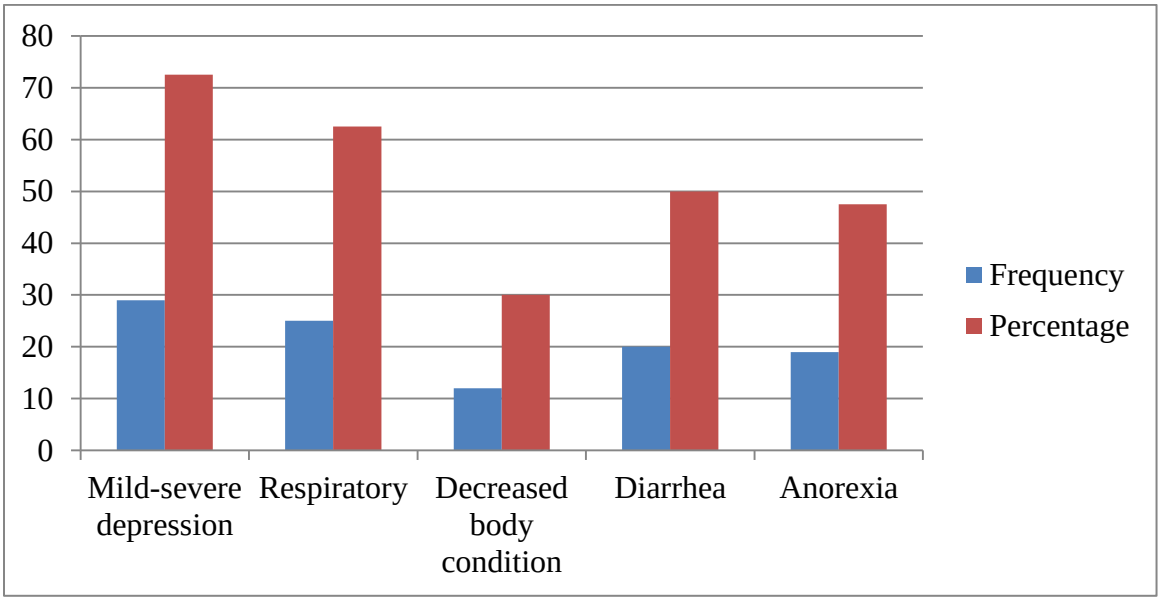


Figure 3: The major clinical signs recorded with their frequencies and percentages

4.2. Gross Lesion Findings

The gross lesions found were recorded with their frequencies thoroughly. The trachea and kidney were the frequently affected organs from all grossly examined organs. Among the examined 40 tracheas, 22 (55%) were found with gross pathological lesions, Thus, some trachea was identified only with the presence of mild to severe hemorrhages **(figure 4A** and **B)** and some were with different amount of clear to turbid mucous exudates inaddition to hemorrhage. On the other hand, Out of 40 examined kidneys, 15 (37. 5%) were observed with gross pathological lesions. The affected kidneys were enlarged, pallor in color and ureters that contain urate deposits **(figure 4D).**

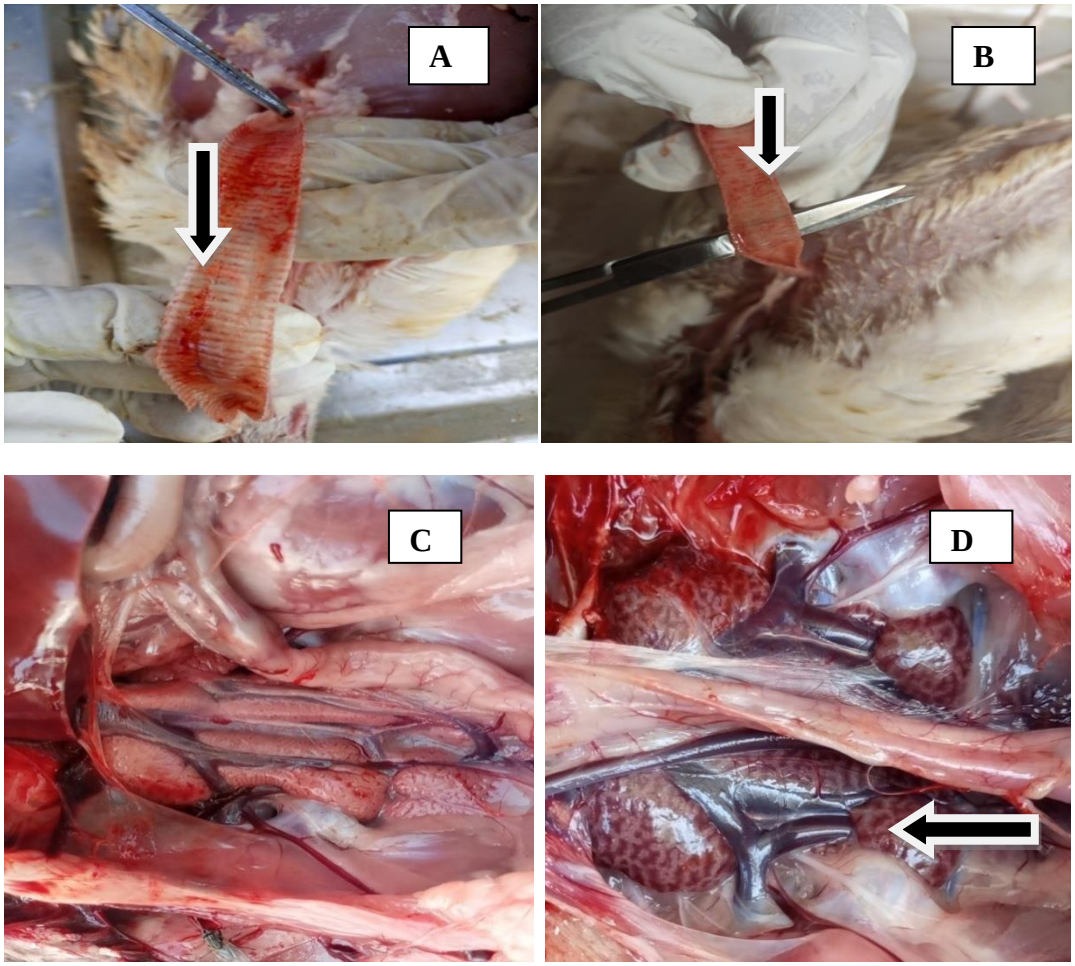


Figure 4: Various gross pathological lesions found in the examined chickens

Keys: A. Showing severe hemorrhagic trachea with mucus. B. Mild hemorrhage in trachea **C.** Pale kidney. **D.** The kidney become swollen with prominent tubules

Table 1: Gross pathological lesions with their frequency and percentage in different organs of clinically ill chickens N=40.

Organs	Macroscopic lesions found	The examined chickens	
		Frequency	Percentage
Trachea	Hemorrhage	16	40%
	Hemorrhagic with exudate	6	15%
Lung	Congested lung	11	27.5%
Kidney	Swollen and Hemorrhagic kidney	15	37.5%

4.3. Histopathological Findings

Histopathologic based examination results in a variety of descriptive lesions. The resultant lesions may be influenced by the virulence of the prevalent strain and its route of introduction.

A. Trachea: The microscopical lesion that found on the trachea collected from IBV positive chicken shows greater impact of IBV on the layers of the trachea especially its mucosal layer. The effect was characterized by loss of epithelial cells with its cilia, infiltration with inflammatory cells mainly lymphocyte, congestion and hemorrhages in the submucosal layer of trachea **(Figure 5).**

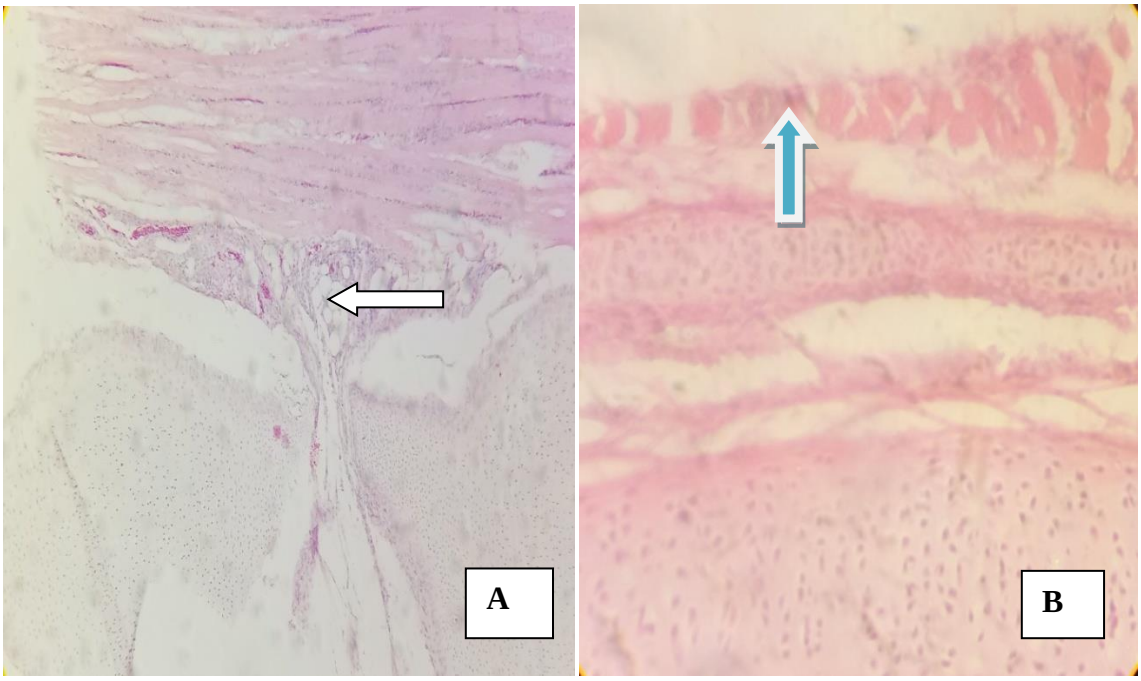


Figure 5: Lesions found within the trachea under light microscope (H and E) 40x and 100x respectively.

Keys: (A and **B)** Sloughed mucosal epithelium blue arrows **(A)** Increased in the numbers of goblet cells indicated by white arrow.

B. Lungs: The microscopical lesion that found on the trachea effect was characterized by the presence of mild to severe congestion were found within the lung (**Figure 6**), H & E 40x.

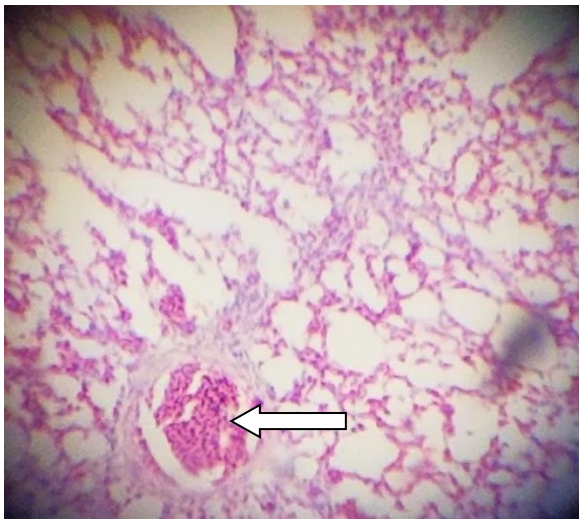
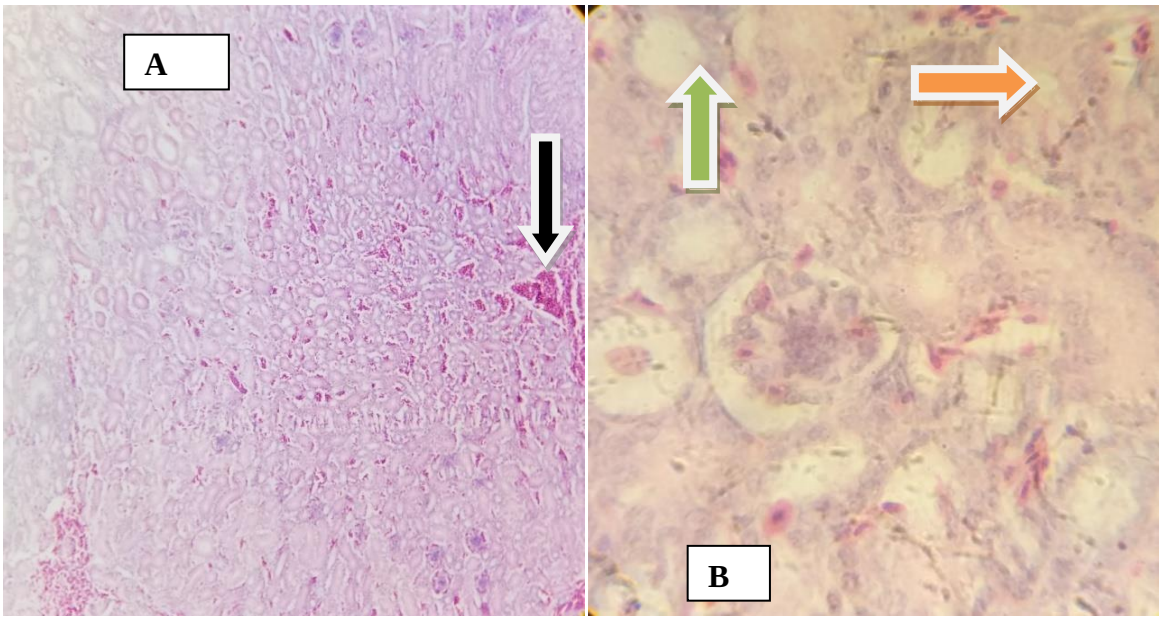


Figure 6:- Microscopic lesions found within the lung

Keys: Congestion **(**white arrow)

C. Kidneys: The microscopic lesions found within the kidney were characterized by the presence of mild to severe congestion and hemorrhages, infiltration of leukocytes, degenerative changes in the renal tubular epithelium, tubular dilation and vacuolation in the tubular epithelial cells (**Figure 7).**



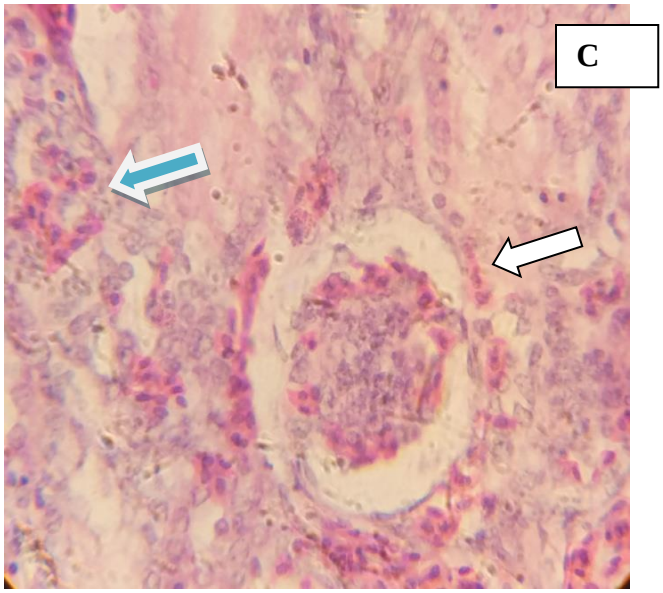


Figure 7:- Pathological lesions present within the kidney (H and E) 40x and100x

Keys: (A and C) Congestion (indicated by black arrow) (40x); **(B).** Degenerative changes in the renal tubule and tubular dilation (red and green arrows, respectively) (100x); **(C).** Infiltration of leukocytes indicated by blue arrow) (100x).

4.4. Molecular findings

RNA extracted from swab samples of all 40 chickens was used to amplify IBV genome using Real Time RT-PCR. Results of amplification were analyzed in comparison with cycle threshold values of positive and negative control. Among the 10 total samples, 8 samples were positive and 2 samples were negative for IBV by real time RT-PCR **(Figure 8).** The real time RT-PCR results revealed that 80% (32/40) of the suspected infected IBV flocks were indeed IBV-positive. From all IBV positive samples, the sample that can pass the threshold line earlier is strongly positive sample. To the inverse, sample that needs more cycle to pass the threshold line is strongly week

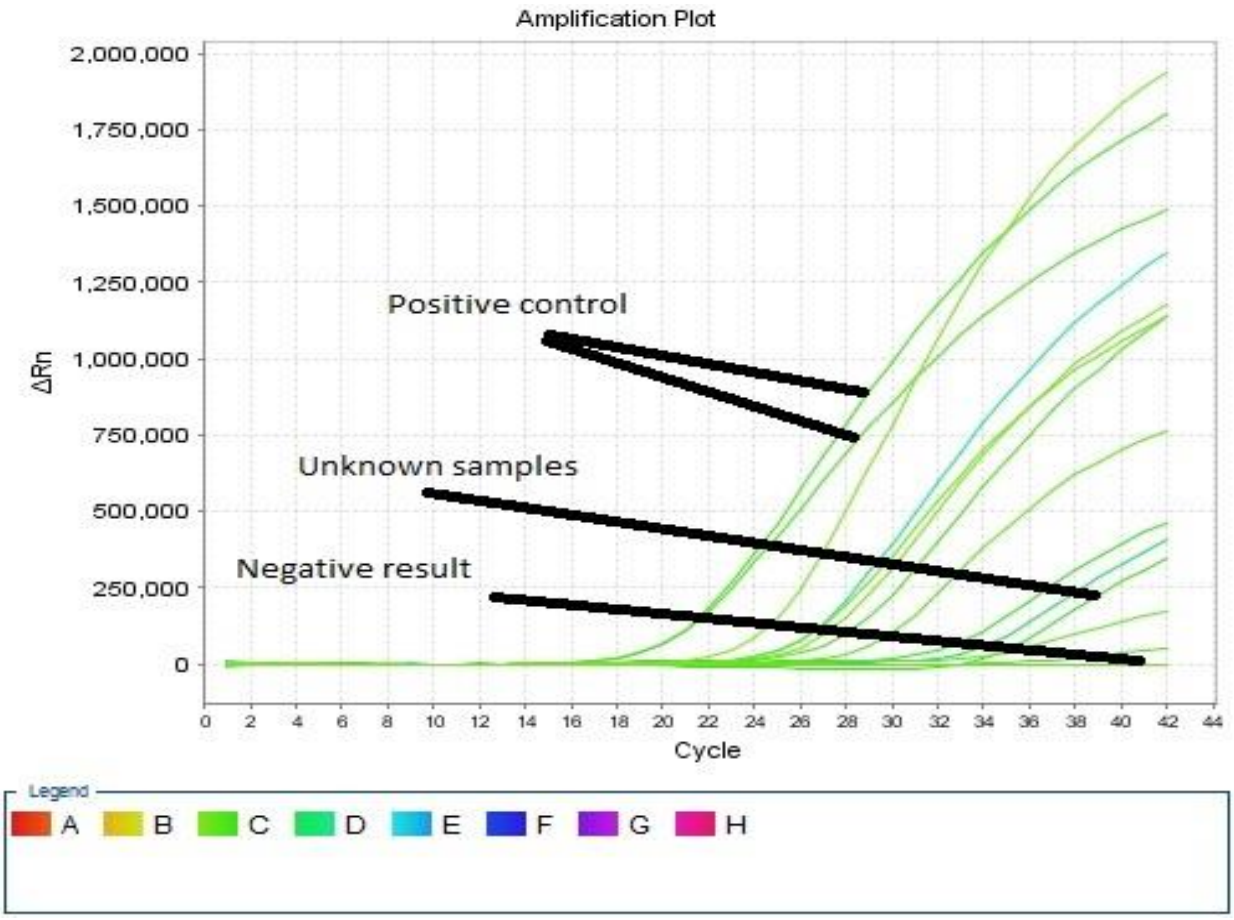


Figure 8: Amplification plot for real time RT-PCR.

5. DISCUSSION

Infectious bronchitis virus (IBV) was the first coronavirus described and sequenced completely. The genomic RNA of coronaviruses is the largest among RNA viruses, approximately 27-30 kb (Lee *et al.*, 2004). The high rate of mutation and recombination are the main characteristics of RNA viruses. This allows them to rapidly adaptation to new environments. Rapid evolution may also hinder the understanding of molecular epidemiology, affect the sensitivity of diagnostic assays, limit the vaccine efficacy and favor episodes of immune escape, thus significantly complicating the control of even well-known pathogens (Legnardi *et al.*, 2020). Despite being known since the 1930s, IBV still estimated as the second most damaging poultry disease after highly pathogenic avian influenza due to its global economic impact (Gallardo, 2021).

For the current study, a total of 40 chickens were examined and characterized by clinical signs, gross and microscopic lesions. Additionally, IBV detection was also performed. Based on the real time RT-PCR detection of IBV, 80% (32/40) of the suspected infected IBV chickens were indeed IBV-positive. This finding is in disagreement to the result of the current study. The tissue samples of IBV positive samples during rRT PCR were then subjected to AH histopathology laboratory for histopathological evaluation.

Among all examined study chickens, predominant lesions were observed in the kidney and trachea organs. This finding was similar to the findings that have been described previously Pourbakhsh *et al. (*2007), Terregino *et al.* (2008), and Gaba *et al.* (2010). The clinical signs recorded in the current study were characterized by coughing, sneezing, dyspnea, nasal discharge, loss of appetite, reduced body weight gain, depression, growth retardation. This clinical finding was supported by Terregino *et al.* (2008), Mahmoud *et al.* (2019) and Bijanzad *et al.* (2013). Of these, depression and respiratory problems were the most frequent clinical signs that represented 93.75 (n= 75) and 96.25% (n=77) respectively as compared with other clinical manifestations. This finding is in agreement with the study of Hasan *et al.*(2020) and Khataby *et al.*, (2016) who stated that the respiratory clinical signs were predominant in all of the inoculated groups and the most prominent clinical signs of IBV were gasping, depression, sneezing, difficulty in breathing, cough and tracheal rales.

In contrast, the current results are different from Tegegne *et al.* (2020) who indicated that clinical signs were not detected in the IBV-positive flocks. The macroscopic lesions observed in the present study were recorded. Among the examined 40 tracheas, 30 (37.50%) were found with gross pathological lesions, Thus, some trachea was identified only with the presence of mild to severe hemorrhages and some were with different amount clear to turbid mucous exudates inaddition to hemorrhage. These gross finding is in disagreement to the result of previous reports by Najimudeen *et al.* (2022) and Grgi *et al.* (2014) who indicated no gross lesions were observed in the trachea, lung, and kidney of birds in any of the groups. Conversely, these macroscopic lesions of trachea recorded in the present study were similarly stated by different researchers (El-Fetouh *et al.,* 2016; Manswr, 2018; Mahmoud *et al.*, 2019).

On the other hand, Out of 80 examined kidneys, 19 (23.75%) were observed with gross pathological lesions. The macroscopic lesions found in the kidneys were characterized by hemorrhages, enlarged, severe congestion and dark red or pale in colour with the presence of whitish precipitate (urates) in the ureters**.** These gross findings were similar with the previous reports of Sediek and Awad (2014), Mahmoud *et al.* (2019), Bijanzad *et al.* (2013) and El-Fetouh *et al.* (2016). The present findings were also consistent with the reports of Boroomand *et al.* (2012) and Lee *et al.* (2004) who observed similar gross lesions in experimentally infected broiler chickens with 793/B IBV serotype and in experimentally infected broiler chickens with CV56b, Gray and Wolgemuth strains respectively. However, according to the report of Khataby *et al*.(2016), gross lesions of kidney were not observed in all experimentally inoculated chickens with Italy 02 IBV serotype. This finding is in disagreement to the result of the current study.

The necropsy findings were followed by histological exanimations of the representative tissues and the major microscopic lesions identified and recorded were characterized by mild to severe congestion and hemorrhages, infiltration of hetrophils, sever hyperplasia leads to thinking in large epithelial areas, deciliation and desquamation of the epithelium of trachea. Previously Pourbakhsh *et al. (*2007) and Hasan *et al.* (2020) were done investigation on the experimental histopathologic study of the lesions induced by serotype 793/B (4/91) infectious bronchitis virus and pathological effect of infectious bronchitis disease virus on broiler chicken trachea and kidney tissues respectively. They report some microscopic lesions that were similar with the

present findings. These histopathological finding was also agrees with the previous investigation of Zhang *et al.* (2017), Okino *et al.* (2017) and Najimudeen *et al*. (2022). Microscopically, the lung was characterized by presence of mild to severe congestion, haemorrhage, oedema with thickening of the interalveolar tissue and infiltration of lymphocytes and few heterophils, severe interstitial pneumonia**.** These microscopic lesions recorded in the present study were similar with the report of Bijanzad *et al*. (2013).

In the present study, the histopathological findings of kidneys were characterized by the presence of hetrophils infiltration, mild to severe congestion and hemorrhages, desquamation and degeneration of tubular epithelium. The same study was done by El-Fetouh *et al.* (2016) about pathological studies on Infectious Bronchitis Disease in chickens and reports some histopathological lesions that were similar with the present findings. Similar results were also recorded perilously by the work of Pourbakhsh *et al. (*2007) and Bijanzad *et al.* (2013)

6. CONCLUSION AND RECOMMENDATIONS

Unhealthiness due to viral, bacterial and parasitic diseases is the main bottleneck of poultry production in the developing countries including Ethiopia. Infectious bronchitis virus is still considered as the second most damaging poultry disease due to its global economic impact next to the highly pathogenic avian influenza. For this reason the present study was focused on the determination of macroscopic and microscopic pathological impacts of infectious bronchitis virus. Infectious bronchitis virus was detected in the swab samples collected from suspected chicken farms. During the examination of the study population, various clinical signs, gross and histopathological lesions were identified and recorded. The overall results of the present study revealed that infectious bronchitis disease is the main cause of health problem in chicken that might be hinders their productiveness and reproductiveness in the study area. This makes the chicken holders to lost more benefits from chickens.

Based on the above conclusion, the following recommendations were forwarded:

- ✓ Implementing of a strong biosecurity measures including vaccination on regular basis might be good to decrease the diseases of poultry.
- ✓ Further study should be done since the virus undergo mutation quickly to identify the strains of the virus which can be helps us to plan the preventing strategies in the future.

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8. ANNEXES

Annex 1: -Chicken necropsy procedures **(Butcher and Miles, 2018)**

Step 1: Obtain the history

Since having good history about the study chickens are necessary for making a correct diagnosis so both individual and flock history should be obtained. The history should include: age, sex and breed of the chickens, clinical signs, disease history, medication history and other information that may be relevant to the case such as type of feed and water. The following information are also required for flock history.

- ✓ Number of birds in the flock
- ✓ Number of birds in the affected group
- ✓ Number of affected birds
- ✓ Clinical signs of the flock should also be noted.

Step 2: External examination of the chicken

The facial tissues, respiration and gait of the chicken should be closely examined for any abnormalities. The comb and wattles for any swelling or discoloration.

Step 3: Open the Body

- ➢ The body should be placed on its back with its feet facing you.
- ➢ Reflect the wings back.
- ➢ Cut through the skin between the legs and the breast so the legs can be fully abducted and lie flat against the table.
- ➢ Remove the skin from the ventral surface of the bird by cutting across at the caudal edge of the keel and then pulling skin cranially and caudally
- ➢ Make a small cut into the body cavity using scissors or a scalpel blade just behind the

breastbone and then pull the abdominal muscle caudally to expose some abdominal viscera.

- Extend the cut up through the cervical area and cut open the beak at the angle of the jaw.
- Now oral cavity, esophagus, trachea and crop are all visible.
- The keel bone and breast muscles are then removed by incising the pectoral muscles on each side of the keel and cutting through the ribs. Use the heavy poultry shears.
- Remove the keel and breast muscles entirely you should now be able to see the internal organs from oral cavity to rectum.
- At this point, all internal viscera should be examined in situ for any abnormalities before removing any organs. Note the color, position and size of all organs and look for any adhesions

Step 4: Remove the Organs and Examine

- It is probably easiest to remove the abdominal viscera first and then go back and remove the thoracic organs
- Remove the liver - in birds the liver takes up a big portion of the abdomen.
- The spleen can be a difficult organ to find once everything is removed so it is a good idea to locate it now and set it aside.
- Pull the proventriculus aside and it should pop into view. Take it out now and set it in a clean dry spot.
- Find the junction of the esophagus and stomach, cut here and pull the digestive tract out, all the way to the cloaca.
- Cut at the end of the large intestine, but leave the bursa in the bird. Kidneys are nestled up against the body wall - there are three portions - cranial, middle and caudal poles.
- In females only the left side of the tract persists, the right is vestigial and too small to be identifiable.
- Pull the heart and lungs away - the lungs are closely adhered to the dorsal body wall, and therefore, careful teasing of the lungs away from the ribs may be necessary to remove them.
- Open up the nasal cavity to take a close look at the sinuses.

- Observe the sciatic nerve which is an important location where Marek's disease can be seen.
- Open some joints to observe the fluid and synovial membranes.

Step 5: Examine and Sample the Organs

1. Examining of the organs

- It is a good idea to go from the "cleanest" organs to the "dirtiest. Usually this order is: lymphoid tissue, brain, lungs, heart, kidneys, reproductive tract, liver, intestinal tract.
- Note any abnormalities for each (color, size, shape, consistency, exudates). Be sure to examine both capsular and cut surface. Make several cuts in each organ and collect specimens for further diagnostic work.

2. Sampling of the Organs

- ✓ Tissues should be collected in duplicate, with half going into 10% formalin for histopathology and half as unfixed tissues that will be used for bacterial culture etc. if necessary.
- ✓ The following tissues should be collected in all cases: lung, liver, spleen, bursa, brain, kidney, intestines.
- ✓ Additionally, any other tissue that might have lesions (skin, adrenal, ovary, heart etc.) should also be collected.
- ✓ Label the tissues that are collected fresh so that the technician performing the tests can determine what each tissue is.
- ✓ Crushing should be avoided by using a sharp blade and a hard surface when taking samples since this may cause artifact histologically.
- ✓ When examining any organ with a mucosal surface (trachea, esophagus, intestine, etc.) care should be taken not to damage or destroy the mucosal surface by rubbing the surface with fingers or instruments.
- ✓ In order to improve fixation of tissues, samples should not exceed 5mm in thickness

and volume of fixative should be at least 10 times the volume of tissue.

- ✓ Fresh samples should be packaged so that they remain cool and to minimize possibilities of leaking.

Step 6: Write the Report

- No necropsy is complete until all findings have been recorded in written form.
- The report should include at least the following information:
- Species, breed, age, sex and history.
- Dead or euthanized?

Annex 2:- Histopathological procedures (Celio, 2011)

The tissue must pass a series of steps before it looks by the examiners under microscope. To achieve this it is important that the tissue must be prepared in such a manner that it is sufficiently thick or thin to be examined microscopically and all the structures in a tissue may be differentiated.

1. **Tissue fixation**: The most commonly used fixative in histopathology is 10% neutral buffered formalin so the first step of tissue processing for histopathology is fixation of tissue samples with 10% neutral buffered formaldehyde**.**
2. **Tissue Trimming:** Trimming is cutting a fixed tissue or organ to create a flat surface suitable for tissue cassettes. Trimming should be done after, not prior to, fixation.
3. **Processing of tissue samples:** The processes starts by fixing tissues with formalin (formalin I and II), dehydrating tissues by soaking them in ascending concentration of alcohols (70%, 95% and 100%), clearing of tissue by xylene (Xylene I, II, III) and impregnation of tissue by paraffin wax (paraffin I and II). The process with the time needed is shown below.

   Formalin-I for 2hr

   Formalin-II for 2hr

   70% Alcohol for 1hr

   95% Alcohol for 1hr

100% Alcohol-I for 1hr

100% Alcohol-II for 2hrs

100% Alcohol-III for 2hrs

Xylene I for 1:30hrs

Xylene-II for 1:30hrs

Xylene-III for 1:30hrs

Paraffin-I for 2hrs

Paraffin-II for 3hrs

4. **Embedding of processed tissue:-** Tissue embedding involves carefully removing processed tissue from the cassettes, placing them into a mold while maintaining their original orientation, and then filling the mold with paraffin wax. **This produces a paraffin block.**
5. **Sectioning:-** Sectioning of paraffin blocks is done using a microtome that cuts very thin (3-5 micron) sections of the paraffin-embedded tissues and put on water bath to straighten the ribbon, and then adhere on the surface of frost ended and clear slide. Later label and put an incubator overnight.
6. **Staining procedure:**

- ✓ Put the sections fixed on slides in xylene (xylene for 5 minutes and xylene for 5 minutes. Then transfer to absolute alcohol (100% for 3 minutes, 100% for 3 minutes and 100% for 3 minutes).
- ✓ Transfer to 95% alcohol for 3 minutes.
- ✓ Place in 70% alcohol for 3 minutes.
- ✓ Rinse the slide in running tape water for 1 minute and put in **Harris‟s Haematoxylin** for 10-15 minutes.
- ✓ Rinse in running tape water
- ✓ Counter stain with **eosin** (3 dips).
- ✓ Rinse in running tape water.
- ✓ Dehydrated in ascending grades of alcohol1 (70 % 3 dips, 95 % 3 dips, 100% I for 3 minutes, 100% for 3 minutes & 100% III for 3 minutes)
- ✓ Cleared it in xylene (Xylene-I for 5 minutes , Xylene-II for 5 minutesϖ & XyleneIII for 5 minutes and mounted with DPX or Canada balsam.

- ✓ Microscopic examination: stained slide is examined under microscope at 4x, 10x, 40x and 100 x magnification for the presence of microscopic lesions and finally photographs of the slides were taken

Annex 3:- Molecular procedures

A. RNA Extraction procedure

1. Pipet 560 µl prepared Buffer AVL containing carrier RNA into a 1.5 ml micro-centrifuge tube.
2. Add 140 µl plasma, serum, urine, cell-culture supernatant or cell-free body fluid to the Buffer AVL–carrier RNA in the micro-centrifuge tube. Mix by pulse-vortexing for 15 s.
3. Incubate at room temperature for 10 min.
4. Briefly centrifuge the tube to remove drops from the inside of the lid.
5. Add 560 µl ethanol (96–100%) to the sample and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.
7. Carefully open the QIAamp Mini column, and repeat step 6. If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.
8. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
9. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 11; or to eliminate possible Buffer AW2 carryover, perform step 10 and then continue with step 11.
10. Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column

and add 60 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.

12. Centrifuge at 6000 x g (8000 rpm) for 1 min.

Annex 4: Ethical clearance certificate