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MOLECULAR IDENTIFICATION OF MAJOR BACTERIAL AND VIRAL PATHOGENS OF CHICKENS AND THE PUBLIC HEALTH IMPORTANCE OF THE PATHOGENS IN COMMERCIAL POULTRY FARMS IN BISHOFTU AND MOJO, CENTRAL ETHIOPIA

Ph.D. Dissertation

BY

BEHAILU ASSEFA WAYOU

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY, PUBLIC HEALTH

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A Dissertation Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Public Health

By

Behailu Assefa Wayou

Advisors

Principal Advisor: GEZAHEGNE MAMO (DVM, MSc, Ph.D., Prof.)-CVMA-AAU

Co-Advisors: TESHALE SORI (DVM, MSc, Ph.D., Prof.)-CVMA-AAU

MATTIA CECCHINATO (DVM, MSc, PhD, Prof.)-MAPS-University of Padova (Italy)

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Submitted by: Behailu Assefa Wayou _____ _____
Name of Ph.D. student Signature Date

Approved for Submittal to Dissertation assessment Committee

1. Major Advisor

Gezahegne Mamo (Prof.) _____ _____
Signature Date

2. Co-Advisor(s)

Teshale Sori (Prof.) _____ _____
Signature Date

Mattia Cecchinato (Prof.) _____ _____
Signature Date

Department Chairperson

Bekele Megersa (Prof.) _____ _____
Signature Date

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Veterinary Microbiology, Immunology and Public Health

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We, the members of the Examining Board of the final Ph.D. open defense have read and evaluated the Dissertation prepared by Behailu Assefa titled: Molecular Identification of Major Bacterial and Viral Pathogens of Chickens and the Public Health Importance of the Pathogens in Commercial Poultry Farms in Bishoftu and Mojo, Central Ethiopia, and recommend that it be accepted as a partial fulfilling of the dissertation requirement for the degree of Philosophy in Veterinary Public Health.

Prof. Bekele Megersa	_____	_____
Chairperson (title and name)	Signature	Date
Dr. Bedaso Mamo	_____	_____
Internal Examiner (title and name)	Signature	Date
Prof. Endrias Zewdu	_____	_____
External Examiner (title and name)	Signature	Date

I hereby certify that I have read the revised version of this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Prof. Gezahegne Mamo	_____	_____
Dissertation advisor (title and name)	Signature	Date
Prof. Bekele Megersa	_____	_____
Department chair (title and name)	Signature	Date

DEDICATION

This Ph.D. dissertation work is dedicated to my father, whom I lost him, at the early start of the Ph.D. program. I am very much indebted to him for every success in my past career. May his soul rests in peace.

STATEMENT OF AUTHOR

This thesis is solely prepared during the accomplishment of the Ph.D. degree where all sources of material used in this thesis have been duly acknowledged. This dissertation is submitted to Addis Ababa University, College of Veterinary Medicine and Agriculture, As partial fulfillment of the Ph.D. degree, and is deposited in the college library to be made available to borrowers under the rules of the Library. No part of this thesis has been submitted in my name to any institution to obtain a degree, diploma, or certificate.

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Name: Behailu Assefa Signature: _____

College of Veterinary Medicine and Agriculture, Bishoftu

Date of Submission: _____

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LIST OF ABBREVIATIONS

AI	Avian Influenza
aMPV	Avian metapneumovirus
DNA	Deoxyribonucleic acid
FTA	Flinders Technology Associates
HA	Hemagglutination
HI	Hemagglutination inhibition
HPAI	Highly Pathogenic Avian Influenza
IBV	Infectious Bronchitis Virus
IBDV	Infectious Bursal Disease Virus
ILT	Infectious laryngotracheitis
LPAI	Low Pathogenic Avian Influenza
MAPS	Department of Animal Medicine, Production and Health
MD	Marek's Disease
NDV	Newcastle Disease Virus
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
spp.	Species

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LIST OF PUBLISHED ORIGINAL ARTICLES

1. **Behailu Assefa Wayou**, Gezahegne Mamo Kassa, Teshale Sori, Alessandra Mondin, Claudia Maria Tucciarone, Mattia Cecchinato and Daniela Pasotto **(2022)**. Molecular Survey and Identification of *Campylobacter* spp. in Layer Farms in Central Ethiopia. ***Trop. Med. Infect. Dis*** . *(MDPI), 7*, 31

2. **Behailu Assefa Wayou**, Gezahegne Mamo Kassa, Daniela Pasotto, Teshale Sori , Claudia Maria Tucciarone , Mattia Cecchinato (**2021**). Molecular survey of viral poultry diseases with an indirect public health significance in central Ethiopia. ***Animals (MDPI),* 11:**3564.

MOLECULAR IDENTIFICATION OF MAJOR BACTERIAL AND VIRAL PATHOGENS OF CHICKENS AND THE PUBLIC HEALTH IMPORTANCE OF THE PATHOGENS IN COMMERCIAL POULTRY FARMS IN BISHOFTU AND MOJO, CENTRAL ETHIOPIA

ABSTRACT

The importance of poultry production is increasing in Ethiopia where high-quality protein and contained costs make poultry a valuable food resource. However, it entails some problems linked to rural, backyard and intensively reared flock proximity and pathogen circulation. The growing poultry production particularly in large-scale commercial intensive systems is challenged by occurrence of diseases of economic and public health importance. This study was planned to investigate the presence of important pathogens in chicken with a direct and indirect public health importance (Campylobacter, Salmonella, Newcastle Disease Virus (NDV), Infectious Bursal Disease Virus (IBDV), Infectious Bronchitis Virus ((IBV), and Avian Metapneumovirus (aMPV)), in poultry farms in Bishoftu and Mojo, Central Ethiopia. Respiratory tract and cloacal swabs and bursa of Fabricious and kidney tissue imprints on FTA cards were collected in 2021 from a total of 500 chickens from 16 farms (1 broiler and 15 layer farms) and tested using PCR-based molecular methods for pathogen detection and sequencing method for species and strain identification. To generate farm-level data, the samples were pooled per farm, shed/flock, and sample type (matrix) where each swab pools contained ten swabs and each tissue smear pools contained variable number of imprints (two to five tissue smear pools). Based on their tropism for specific body system/part or tissue, twenty cloacal pooled samples were tested for Campylobacter and Salmonella species identification using genus-specific PCR. On the other hand, forty cloacal and respiratory swab pooled samples, and fourteen bursal and kidney tissue imprint pooled samples were tested using reverse transcription PCR, for different viral agents. Among the total twenty cloacal pooled samples tested, 70% (14/20) of them were positive for Campylobacter spp., where 71.4% (10/14) of the positive samples belonged to Campylobacter jejuni species, 21.4% (3/14) belonged to Campylobacter avium and 7.1% (1/14) to Campylobacter helveticus. But, all the twenty cloacal swab samples tested for Salmonella spp. became negative. On the other hand, among the total 16 farms tested for viral pathogens, one farm was positive (6.7%) for NDV

(among 15 layer farms tested for NDV) with a Lasota vaccine strain, genotype II; another one farm was positive (6.25%) for IBDV (out of a total of 16 layer and broiler farms covered in the study), resulting in strains similar to those present in vaccines, Winterfield-2512, belonging to genogroup A1a; two farms were positive (12.5%) for IBV, resulting in a 4/91-like strain/793B (GI-13 lineages); but there were no farm tested positive for aMPV. In this study, Campylobacter jejuni was a predominantly isolated (71.4%) Campylobacter species in chickens in the study area, whereas species such as C. avium and C. helveticus were newly reported in Ethiopia, revealing a variability that needs to be monitored in light of the public health significance of this pathogen. On the other hand, the present findings suggest a low presence of viral pathogens (3/16,18.75% farm and 6/54,11.11% sample pool) probably due to the implementation of vaccination strategies, which is also testified by the detection of vaccine strains. The detection of high total prevalence of pathogens (35.2% (19)) for sample pools, and 81.2% (13) for farm), and particularly of a public health important campylobacter jejuni which has a high zoonotic importance, can imply high transmission potential of the pathogens from poultry host to human being indicating high risk of acquiring the infection. The high rate of detection of an important zoonotic pathogen, campylobacter (70% sample pools), in this study with wider farm coverage (80% farms) and among different host factors, management conditions, vaccination protocol and treatment schemes used, coupled with the poor biosecurity practices encountered during the field data collection, suggested a high risk of pathogen introduction to human population and greater dissemination potential. It was the limitation of laboratory facilities and budget related constraints that hinder further extensive investigation of the problems in both chicken and human population, covering wider areas and farms and coming up with detection of the circulating pathogens at their presence and at the same time investigating other public health important zoonotic pathogens of poultry like avian influenza virus and E.coli bacteria. In the future, extensive PCR-based detection of important pathogens circulating among poultry and human population should be carried out to have a clear epidemiological picture of distribution of these pathogens and to let design an appropriate intervention measures for control and prevention of the pathogens.
Keywords: aMPV**,** campylobacter; chickens, IBV; IBDV; NDV; poultry; public health, salmonella

1. INTRODUCTION

The importance of poultry production is globally increasing. This is also true in Ethiopia, where high-quality protein and contained costs make poultry a valuable food resource. In Ethiopia, the vast majority (90.87%) of domestic fowl are reared in villages, under a traditional scavenging system (CSA, 2021) that allows poultry to be owned by almost 60% of households (Wilson, 2010), and only a small percentage (9.11%) are reared intensively (CSA, 2021), although this type of production is consistently increasing. To sustain intensive farming in Ethiopia, exotic breeds are becoming more and more commonly raised. However, this entails some problems particularly being linked to rural, backyard, and intensively reared flock proximity and pathogen circulation. At the same time, large populations of intensively reared chickens are surrounded by small farms and backyard flocks where biosecurity measures are not strict enough, animals of different ages are kept together or birds are not fully vaccinated due to costs, required expertise, and the difficulty of purchasing vaccines for private owners (Mazengia, 2012). This lack of an organized poultry health service and limited access to veterinary assistance, including both diagnosis and vaccination, are inevitably linked to high disease occurrence and mortality rates in birds (Asfaw *et al.*, 2021). The higher host susceptibility together with the possible pathogen introduction along with the new breeds further complicate the disease scenario, due to which there would be suboptimal growth and low productivity levels (Mazengia, 2012). In general, under the different poultry management conditions, diseases, particularly infectious diseases, are the top priority problems and poultry production has been hindered by these diseases (Dereje, 2019).

Poultry pathogens not only impact productivity and animal health but they are intimately connected to food safety and human health, especially when chickens and people share the same environment. Close contact with animals is crucial for transmission of zoonoses. Zoonotic pathogens can spread to humans through any contact point with domestic, agricultural, or wild animals. These pathogens can specifically be transmitted through inhalation, ingestion, conjunctiva, or physical contact. In general, close contact with animals is a risk factor for zoonotic disease transmission (Gijs *et al.*, 2016).

Poultry sometimes carries harmful germs that can spread to people and cause illness, even though, it provides meat and eggs that are nutritionally beneficial food containing high-quality protein (Asfaw *et al.*, 2021). Exposure to animals was indeed proven as one of the major risk factors for diarrhea in children under 5 years of age in Ethiopia (Lengerh *et al*., 2013).

Studies have shown that most of the small-scale semi-intensive poultry farms that are flourishing during the last few decades in urban and peri-urban areas of Ethiopia are located near human residential areas. This suggests the possibility of transmission of potential pathogens to humans. Some of these smallholder poultry producers keep chickens in the same compound where they live, risking the family members to the transmission of zoonotic pathogens (Betelhem *et al*., 2020). Such situations further aggravate inter-species transmission of pathogens at the interface of poultry and the human population.

Bacterial diseases like Campylobacteriosis and Salmonellosis are generally known to hinder poultry production causing serious morbidity and mortality in chickens. In addition, these two diseases are leading foodborne zoonoses in Ethiopia (Lengerh *et al*., 2013; Mulatu *et al*., 2014). They are often the cause of gastrointestinal infections in humans, sustaining serious diseases in children. Immunosuppression, malnutrition, and old age are risk factors for human infection with these bacteria (Tadesse, 2014). In most cases, campylobacteriosis and salmonellosis are self-limiting infections; however, in immunocompromised or critically ill patients, antimicrobial therapy may be required (Kist, 2002).

As a matter of fact, among the most common foodborne zoonotic agents, *Campylobacter* spp. is a bacterium that commonly inhabits the gastrointestinal tract of many domestic animals, especially chickens (Ghoneim *et al*., 2021). Even though Campylobacter is highly prevalent in commercial, and free-range poultry farms, birds that access an environment where no disinfection can be applied and that are reared for longer periods before slaughtering are at higher risk (Heuer *et al*., 2001); as in backyard flocks. There, they can be exposed to wild birds, which are also reservoirs of *Campylobacter* spp., although with

a lower prevalence likely linked to the sparse animal density. Flock size, house number, the use of untreated water, and disposal of poultry wastes on the farm are other risk factors for *Campylobacter* spp. colonization (Hagos *et al*., 2021).

Although poultry acts just as a carrier of *Campylobacter* without clinical symptoms, they can serve as a potential source of human infection (Hagos *et al*., 2019). Among bacterial zoonotic agents, *Campylobacter* spp. is the most important food safety hazard, and together with *Salmonella* spp., they account for more than 90 percent of all reported cases of bacteria-related food poisoning (EFSA, 2021). In Ethiopia, the prevalence of *Campylobacter* spp. infection in poultry ranges from 13.0% to 100% (Hagos *et al*., 2019; Chala *et al.*, 2021*)*

Salmonella spp. is the second most important (next to campylobacter) zoonotic cause of gastrointestinal disease in humans (EFSA, 2021) and could be encountered through contaminated food, mainly poorly cooked meat, and eggs, or water and contact with droppings or animals, that can be asymptomatic reservoirs (Cosby *et al*., 2015). Many factors favor enteric colonization of salmonella in poultry, such as the bacterium resistance to the gastric passage, the age of the birds, the use of antimicrobial drugs, farming conditions and stressors, health status, and immunosuppression. Additionally, for *Salmonella* spp., when introduced into a flock, a high percentage of animals are rapidly colonized. The prevalence of salmonellosis in poultry in Ethiopia can be as high as 15.12% (Abda *et al*, 2021). In Ethiopia, *Salmonella* spp. was recently detected in different animal products (Ejo *et al*., 2016; Eguale, 2018; Ketema *et al*., 2018) at a 17.9% rate (Eguale, 2018), with a parallel increase in antimicrobial resistance against most commonly used drugs (Eguale *et al*., 2015; Eguale *et al*., 2016; Eguale *et al*., 2017; Eguale *et al*., 2018), both in animals and humans.

Viral agents are another most frequently occurring pathogens, especially in Ethiopia. The viral diseases, Newcastle disease (ND), and infectious bursal disease (IBD) are some of the major causes of morbidity and mortality in poultry (Degefa *et al*., 2004; Mazengia, 2012; Hutton *et al*., 2017; Tegegne *et al*., 2020). These are high-priority viral poultry diseases in

Ethiopia, since intensive poultry farming is growing, it is still flanked by rural and backyard flocks, which greatly differ in their health standards and rearing conditions (Mazengia, 2012). Despite the routine vaccinations being implemented on commercial poultry farms in Ethiopia, different viral disease outbreaks have been reported and the mortality rate remains high (Alemu *et al*., 2008).

Newcastle disease (ND) is identified as a major killer, largely contributing to economic losses for the poultry sector in Ethiopia, and it is usually the first disease suspected during disease outbreaks (Habte *et al*., 2017). The prevalence range of NDV in poultry in Ethiopia is between 0.007% (Bettridge *et al*., 2014) and 100% (Solomon & Abebe, 2007). Newcastle disease virus (NDV) can infect humans resulting in mild flu-like symptoms or conjunctivitis (an eye problem that is also called pink eye). In humans, NDV also causes laryngitis (irritation and swelling of the voice box and the area around it) (National Cancer Institute, 2013).

Infectious bursal disease virus (IBDV) is another common viral pathogen that usually affects young chickens and weakens their immune system, predisposing the birds to vaccination failure and opportunistic pathogens, of which some could have zoonotic importance (Rautenschlein and Alkie, 2016). Infectious Bursal Disease Virus is an emerging disease in Ethiopia, and it was detected in 2002 for the first time (Zeleke *et al*., 2005). Its circulation seems to be worsened by the adoption of exotic breeds of chickens, which are considered less resistant than indigenous breeds (Mekuriaw *et al*., 2017; Hinsemu *et al*., 2018). The prevalence of IBD in poultry in Ethiopia ranges from 3.6% (Bettridge *et al*., 2014) to 100% (Solomon and Abebe, 2007) and the mortality rates in Ethiopian chickens due to IBDV were reported to reach 50% (Zeleke *et al*., 2005). Low biosecurity standards contribute to the spread of IBDV. Risk factors such as visitors from different poultry houses, workers owning rural birds, and vendor vehicles, in the presence of low biosecurity standards, aggravate the transmission of the virus (Tulu, 2019). From the few commercial poultry farms situated in the central part of Ethiopia, in which the disease was first reported, IBDV has widely spread to other parts of the country (Berhanu

et al., 2018). Despite regular vaccination practices, IBDV is common in Ethiopia, involving both commercial and backyard poultry (Mekibib *et al*., 2019).

Avian infectious bronchitis (IB) is also an important disease of poultry that affects the respiratory tract, gut, kidney, and urogenital and reproductive systems of chickens (Ignjatovi´c and Sapats, 2000). Few studies conducted in different parts of Ethiopia have reported IBV seroprevalence to be high on both commercial and backyard chicken farms. The range of prevalence of IB in Ethiopian poultry is 6% (Tegegne *et al*., 2020) to 97% (Jirata *et al*., 2022).

Avian Metapneumovirus (aMPV) is another important respiratory pathogen whose importance is growing in poultry. In Ethiopia, aMPV subtype B has been detected both in backyard and intensively raised flocks, with respiratory signs and a prevalence of 21.9% (Hutton *et al*., 2017; Tegegne *et al*., 2020). Vaccination for aMPV is not commonly adopted in chickens, especially in Ethiopia, where it is currently unavailable (Hutton *et al*., 2017), so the control of this agent mainly relies on biosecurity. The introduction of aMPV has also been tentatively linked to the importation of birds (Tegegne *et al*., 2020). Mortality rates are usually low, except for cases of secondary infection that can result in severe forms, in particular with *Escherichia coli* (Suarez *et al*., 2020).

These viral infections in poultry deeply affect production but they can also lead to a greater risk for human health, due to the increased susceptibility to both viral and bacterial secondary infection. For example, IBDV does not have any zoonotic potential, but its immunosuppressive nature could favor the replication of pathogens of zoonotic importance, such as *Salmonella* spp., *Campylobacter* spp., and Avian influenza virus (Subler *et al*., 2006; Eterradossi and Saif, 2013).

Few data are available, particularly on *Campylobacter* spp. presence, their serotypes or biotypes, and prevalence in chickens in Ethiopia. Regardless of the dynamism in the ecology and epidemiology of *Campylobacter* in the environment, chickens, and people, that may drive the emergence of new epidemiological patterns of the disease, the

investigations made so far did not match this dynamism. A study on amino acid sequence alignment also revealed a mutation in the chicken campylobacter isolates (Ghoneim *et al.*, 2021). This further calls for a regular checkup for field isolates circulating among the poultry population in the country.

The same situation happens true regarding salmonellosis in chickens in Ethiopia. The studies on avian salmonellosis in Ethiopia indicated the existence of a high prevalence of Salmonella infection in the country (Abda *et al*, 2021; Belachew, 2021; Betelhem *et al.*, 2020; Eguale, 2018; Mintesnot *et al*, 2011). Different serotypes of *Salmonella* spp. were isolated at different prevalences in chickens in Ethiopia (Belachew, 2021; Eguale, 2018), but those studies have recommended further analysis being demanded.

Similarly, viral diseases are not well studied in most developing countries like Ethiopia (Hutton *et al*., 2017), and the few existing studies in Ethiopia were largely based on serological tests (though there were few molecular-based studies), rather than on molecular characterization of the circulating strains (Damena *et al*., 2016).

All these scarce epidemiological bits of knowledge limit the attempt to control these diseases and the growth of poultry production. The regular investigation, particularly focusing on the usage of genomic sequencing method for characterization of both bacterial and viral pathogens of poultry circulating in the country, for that matter, in the study area is mandatory, because, the current study area is a potential poultry-producing and distributing area in the country. So, it should get attention.

Objective

General Objective

- To molecularly identify pathogens of chicken with direct and indirect public health importance circulating among commercial poultry farms in Bishoftu and Mojo, Central Ethiopia, and to analyze their epidemiology and assess their public health importance.

Specific objectives

- To detect the *Campylobacter* and *Salmonella* genus in the poultry cloacal swab samples using genus-specific PCR targeting the 16S rRNA gene (*campylobacter*) and ; Chromosomal invA (*salmonella*)
- To identify *Campylobacter* and *Salmonella* species in the PCR positive samples by using genomic sequencing technique followed by phylogenetic tree reconstruction;
- To detect viral pathogens: NDV, IBDV, IBV, and aMPV in the poultry samples using one-step RT-PCR assays;
- To characterize the strains of NDV, IBDV, IBV, and aMPV using Sanger sequencing method and further perform the phylogenetic analyses of the pathogens;
- To analyze the epidemiology of the molecularly characterized isolates;
- To assess a public health implications of the identified agents.

2. LITERATURE REVIEW

2.1. An Overview of the Ethiopian Poultry Sector

Since poultry is an extremely valuable food resource, its production is increasing worldwide. This is also the case in Ethiopia. Poultry is the most populous species (57 million) (CSA, 2021) among domestic animals in Ethiopia. Traditional production practices dominate poultry production in the country (Fenet and Alemayehu, 2019). Chickens are the dominant bird type domesticated in Ethiopia and whenever the word poultry is used, it is usually referred to as chickens. Indigenous breeds of chickens are the dominant breeds kept in the Ethiopian rural setting (Table 1). Indigenous breeds of chickens are kept by most of the peri-urban and urban communities of Ethiopia, besides the rural communities. These indigenous breeds of chickens are well adapted to the environmental condition in Ethiopia and that is why they are kept by most of the communities in the country. Even if the country owned a huge chicken flock, the gain from this sector is not to the expected level. To this effect, some decades in the past, the Ethiopian Ministry of Agriculture has decided to introduce exotic breeds of chicken into the country and has planned to increase the number of poultry farms along with proper handling in terms of their management. This is by taking into consideration that poultry farming can solve food security issues in the country (Tewodros and Mebrate, 2019).

Since the introduction of the first batch of exotic breeds of chickens to Ethiopia, that is dated back to the early 1950s (Matawork, 2016b), the importation of exotic breeds of chicken become widely practiced. Thus, many exotic breeds of chicken (White and Brown Leghorns, Rhode Island Red, Bovans, New Hampshire, Cornish, Australorp, and Light Sussex) are introduced into the country (Bayesa, 2021). Nowadays, many of these chicken breeds are distributed to smallholder farmers in different parts of the country.

In Ethiopia, a program of crossing local breeds with the imported exotic breeds, went hand in hand with importing exotic breeds. This is with the intention to improve the performance of the local chickens by increasing the genetic potential of indigenous breeds. Even though

the total number of indigenous breeds still dominates, following the government policy of introducing exotic breeds of chicken and also crossing them with local breeds, the number of indigenous breeds in Ethiopia is decreasing year after year due to their replacement by exotic breeds (Table 1).

Table 1. Total estimates (in million) and percentage of each breed of poultry in Ethiopia (reports of Central Statistical Agency)

Year*	Total No (10^6)	Indigenous (%)	Hybrids (%)	Exotic (%)	Reference
2021	57	78.85	12.02	9.11	CSA, 2021
2017	56.06	88.19	5.36	6.45	Bayesa, 2021
2017	56.53	94.3	3.21	2.49	Tewodros and Mebrate, 2019
2015	56.87	95.86	2.79	1.35	Mekuriaw *et al*., 2017
2007	34.2	94.4	3.92	0.64	Dawit *et al.*, 2008
2005	30.9	97.8	-	2.2	Paolo and Abebe, 2008

* Year reported by CSA, Ethiopia

Despite the government's effort to modernize poultry production by introducing exotic breeds and encouraging more productive technologies (Paolo and Abebe, 2008), their productivity remains much minimal. The production performance of Ethiopian local chicken is relatively lower than the performance of exotic breeds. This could be mainly due to less care given to the local breeds. Would local chickens be given care as exotic breeds; the increased input will increase the output expected from local chickens. But as local breeds didn't get attention in Ethiopia, the egg production potential of local chicken is 30-60 eggs /year/ hen with an average egg weight of 38 g under village management conditions. The exotic breeds rather produce around 250 eggs/ year/hen with an egg weight of around 60 g under an intensive production system (Matebneh, 2019). But the cumulative advantage of exotic breeds is not significantly higher than the local breeds if the local breeds will be kept under intensive management. It is difficult to compare the performance of local breeds kept under backyard management with exotic breeds kept under intensive management. Generally, the overall productivity of the Ethiopian poultry sector is not as per the first expectation of the Ministry of Agriculture while launching the introduction of

exotic breeds and crossing them with local breeds. There are some aspects in which local chickens are preferable over exotic chickens under the current Ethiopian situation. Local chickens can survive well under low input, unlike exotic breeds and they are also resistant to diseases to which exotic chickens are highly susceptible and be affected with. Exotic breeds are susceptible to diseases and they are more probably infected before import (Dereje, 2019).

Among the diseases to which the poultry sector in Ethiopia is much vulnerable, most or all of the diseases are believed to be introduced concurrently with the exotic breeds and exacerbated by the intensification of the poultry industry. In addition to the exotic breeds in the intensified commercial farms, the growing number of exotic flocks in the backyard system increase the number of birds that are at risk of getting infected with exotic pathogens and with pathogens in the environment (Mazengia, 2012). The distribution of improved breeds of chickens from infected poultry breeding and multiplication centers to the village is also suspected of disseminating diseases to indigenous chickens reared by the farmers (Hailu *et al*., 2009).

Besides diseases, factors like predators, lack of proper healthcare provision, scarce feed resources, and poor marketing information hinder the productivity of the chickens in most areas of Ethiopia. Among those obstacles, diseases are the main constraints incriminated for the reduction of total numbers of poultry and compromised productivity (Tewodros and Mebrate, 2019). As reported by Dawit *et al*. (2008), 62% of the respondent smallholder farmers reported disease as the major factor for the high mortality in local birds. So, it is critically important to develop methods to expand poultry farms while lowering the prevalence of infectious diseases that inflict significant losses each year (Dereje, 2019). It should be ensured that the exotic birds are free of disease and also have enhanced resistance to key infectious diseases before importation and good biosecurity measures are in place during importation and throughout the farming practices.

2.2. Major Poultry Diseases in Ethiopia and Associated Risk Factors

In Ethiopia, poultry production has been hindered by different prevalent diseases. Newcastle disease, coccidiosis, salmonellosis, and chronic respiratory disease (Mycoplasmosis) were reported to be the prevalent diseases in Ethiopia that hinder poultry production (Solomon, 2006). In addition to the above diseases, Yohannes *et al*. (2019) reported Infectious Bursal disease, helminths, ectoparasite infestation, and Campylobacter infections as the most important diseases of chickens in Ethiopia based on their prevalence and expected impacts they impose on poultry productivity. These and other prevalent diseases affect chickens under all the production systems that range from the village to the commercial production systems. Newcastle disease, salmonellosis, avian cholera, coccidiosis, and avian pox are diseases to which chickens raised under village production systems are exposed (Bettridge *et al*., 2014) and are the major causes of morbidity and mortality in village poultry (Tadiose *et al*., 2017). Similarly, gastrointestinal helminths, Marek's disease, and Newcastle disease are the prevalent poultry diseases in large commercial poultry farms in Ethiopia. In small-scale farms, it is coccidiosis which is the main disease. Avian flu can be equally detrimental to the poultry sector in Ethiopia. It has a greater knock-on effect on poultry production and public health with possibilities of transboundary transmission and the potential to transmit to humans (Dawit *et al*., 2008).

Like the zoonotic avian influenza, some of the prevalent poultry diseases can also spread to humans via the consumption of poultry meat and eggs or contact with infected birds and contaminated materials. Even though poultry meat and eggs provide nutritionally beneficial food containing protein of high quality, they may present some risks for consumers. This is because they may be infected by bacterial infections such as Salmonella and Campylobacter (Teferi, 2016).Raw chicken is often contaminated with Campylobacter bacteria and sometimes with Salmonella and *Clostridium perfringens* bacteria. Campylobacter prevalence is higher in chickens and their meat compared to other farm animals and meat products (Brena *et al.*, 2016). *Salmonella* Typhimurium and *Salmonella* Enteritidis are also the most common agents of foodborne disease in humans (Tadiose *et al*., 2017).

There are different factors and human activities that have aggravated the magnitude and distribution of significant poultry diseases in Ethiopia. In fact, there are no risk-free practices available in the country that don't have played a role in the introduction of exotic diseases and transmission of infectious poultry diseases from infected initial farms to particular disease-free farms. All the practices (from importation-related activities to specific poultry farm management practices) have an associated risk of introducing infection agents from abroad into the country and distributing the agent among farms in the country. This is because, due to the high demand for poultry products and by-products that calls for huge market access, a lot of the public is participating in poultry farming. The problem is that most of these poultry keepers are business-driven, motivated by the money they can fetch from the business, and they lack expertise in risk-free management of the operation. That is, some of them manage poultry at a standard lower than the way local farmers who have gained indigenous knowledge by keeping poultry over the years, keep their poultry. However, there is a trend of shifting from keeping indigenous poultry breeds or at least crossbreeds to keeping exotic breeds without any thorough investigation into their adaptation and drawback. All these have footings in bringing poultry diseases home (Paolo and Abebe, 2008).

In Ethiopia, there is uncontrolled importation of poultry particularly day-old chicks from abroad as well as movement from farm to farm. Government-owned poultry multiplication centers throughout the country, non-governmental organizations, and private individuals distribute these chickens to smallholders. These close links between intensive and smallholder farms facilitate disease spread, exacerbated by low biosecurity, and poor access to veterinary inputs and expertise among smaller producers (Hutton *et al*., 2017). These could create a high chance of the introduction of exotic diseases and increased distribution of the existing ones among different poultry farms and areas in the country. These were how an opportunity has been created for the introduction of exotic viral and bacterial poultry diseases into the country and their transmission from farm to farm as well as the passage of the endemic viruses and bacteria from one area to another area. The intensification and dissemination of the exotic breeds and their crosses to villages have also

created chicken populations that are susceptible to major viral and bacterial diseases (Mazengia, 2012). The free movement of backyard poultry between families in the Ethiopian village could also contribute to the transmission of many infectious diseases in the backyard system (Dawit *et al*., 2008). The movement can be from household to local market for sale, from market to household in case of unsold chicken, or the form of gifts from household to household.

Following these perspectives related to poultry management and trade, foodborne diseases became one of the most widespread global public health problems of recent times, and their implication for health and the economy is increasingly recognized. Poultry is a common source of human infection, and *Campylobacter* and *Salmonella* are common bacterial pathogens in this regard as they are transmitted from poultry and poultry products to humans (Betelhem *et al*., 2020).

2.3. Major Bacterial Poultry Diseases in Ethiopia

Campylobacter and Salmonella infections were found to be the most important diseases of chickens in Ethiopia based on prevalence (Yohannes *et al*., 2019). These diseases appear to have serious impacts on poultry productivity. They can cycle through a flock when older birds that show no symptoms pass the illness to younger birds. Every new generation of bacteria will be deadlier than the last and can endanger an entire flock. In addition to their problem in poultry production, Campylobacter and Salmonella can contaminate raw chicken and be a source of human infection, though chicken is a nutritious choice for human consumption. Campylobacteriosis as a clinical disease is not common in poultry and other birds but it is a significant enterocolitis in people, frequently acquired through consumption of undercooked poultry meat contaminated with campylobacter, particularly *Campylobacter jejuni* (Facciola *et al*., 2017).

2.3.1. Campylobacteriosis in Poultry

Changes to the system of chicken production have altered the ecology and epidemiology of *Campylobacter* in the environment, chickens, and people, which may drive the emergence of new epidemiological patterns of the disease. An amino acid sequence alignment study revealed a mutation in the chicken campylobacter isolates that have zoonotic significance (Ghoneim *et al.,* 2021) and the strains isolated from chickens have been observed to correlate with human isolates in terms of biotype and serotype (Brena *et al.*, 2016).

Flock size, the number of houses, use of untreated water, and disposal of poultry wastes on the farm increase risks of *Campylobacter* spp. colonization of poultry flocks (Jay *et al*., 2008). IBDV infection also exacerbated colonization and shedding of Campylobacter, particularly *C. jejuni*, likely through the immune suppression this virus causes in chickens. The disease's seriousness relies upon the virulence of the strain and on the host's immune state (Subler *et al*., 2006).

In Ethiopia, it was reported that chickens and their meat have a higher *Campylobacter* spp. prevalence compared to other farm animal and their meat products (Brena *et al.*, 2016). Birds act as a source of infection through direct and domestic exposure (Lengerh *et al*., 2013). Poultry workers in farms, slaughterhouses, and meat processing facilities are at risk of *Campylobacter* and *Salmonella* species infections (Garedew *et al*., 2015) through direct contact with infected birds, their products, and contaminated materials or environments. Studies on the prevalence of Campylobacter in Ethiopia have identified a range of 13.8–50.0% in humans, and 10.6–56.5% in animals (Chala *et al*., 2021). A systematic review and meta-analysis conducted by Zenebe *et al*., 2020 on 12 articles published from 2004 to 2020, showed that the pooled prevalence of *Campylobacter* species from different sources was 10.2% (6.0% to 72.7%). In this meta-analysis, it was found that the prevalence of *Campylobacter* was higher in animals (14.6%) compared to humans (9%). Desalegne and Adane, 2010 also reported that the overall prevalence of thermophilic campylobacters was 8% and 72.7% in humans and chickens, respectively. The other thing is that, once

Campylobacter is introduced into a flock, the prevalence reaches up to 100%, and up to 100 % of broilers at slaughter age may harbor the organism (Hagos *et al*., 2019). Also, look at the Campylobacter prevalence in animals through the years in Table 2 below.

Table 2. Prevalence of Campylobacter based on fecal and meat samples collected from chickens (*C. jejuni, C. coli, C. lari*, and *C. fetus*)

Location	Sample	Testes conducted	No tested	Overall prevalence	Reference
Addis Ababa	Feces	PCR	69	13.0	Chala *et al., 2021*
Horro, Jarso and Debre Zeit	Feces	polymerase chain reaction (PCR)-specific assays	44	18.4	Brena *et al*., 2016
Bahir Dar	Feces	Culture	220	72.7	Desalegne and Adane, 2010
Addis Ababa and Debre-Zeit	Meat	Culture method	60	21.7	Lemma and Daniel, 2009
Jimma	Feces	Culture method	191	68.1	Tesfaye *et al*., 2005

2.3.2. Salmonellosis in Poultry

Domestic poultry constitutes a large single reservoir of salmonella organisms existing in nature. Based on prevalence, Salmonella infections were found to be the most important disease in chickens. Salmonella can cycle through a flock when older birds show no symptoms or the ill birds pass the agent to younger ones (Agnieszka and Katarzyna, 2018). Chickens are the natural hosts for the highly host-adapted biovar *S.* Gallinarum and *S.* Pullorum. In many developing nations, *S.* Pullorum infections of poultry are common and Pullorum disease remains among the principal disease threats to poultry producers (Netsanet *et al*., 2012).

Salmonella serovars that are common in poultry vary in their spatial distribution. Both the host unrestricted serotypes of avian Salmonella; *Salmonella* Enteritidis and *Salmonella* Typhimurium, and the host restricted serotypes that are common to poultry; *Salmonella* Pullorum and *Salmonella* Gallinarum, are found worldwide. Other *Salmonella* serotypes are limited to specific geographic regions (Eguale, 2018).

In Ethiopia, the history of salmonellosis and the development of industrial poultry breeding are associated. With the great expansion of the poultry industry, there is an associated widespread occurrence of avian salmonellosis that put it to be ranked as one of the most important egg-born bacterial diseases of poultry. The few studies on avian salmonellosis in Ethiopia indicated the existence of a high prevalence of Salmonella infection in the country. In Ethiopia, there is fowl typhoid and pullorum disease in both exotic and local breeds, as indicated by the seropositivity and isolation tests conducted for Salmonella organism detection. The detection of salmonellosis with high overall prevalence and a higher proportion of those with likely zoonotic potential and multi-drug resistant isolates, indicated that salmonellosis could be an emerging poultry and public health problem in Ethiopia. Furthermore, the finding that Salmonella was isolated from different sample types, poultry growth stages, and breeds indicates its wider distribution in Ethiopia. The existence of the diseases especially in the local breeds of Ethiopia (ecotype: Cheffe, Jarso, and Horro) is of great concern as the diseases have the potential for horizontal and vertical transmission (Yizengaw, 2015; Abunna *et al*., 2016; Betelhem *et al*., 2020).

Several hosts of unrestricted *S. enterica* serovars were frequently isolated from poultry without showing any clinical signs. Three serovars (i.e., S. Haifa, S. Anatum, and S. Give) all belonging to the *Salmonella enterica* subspecies Enterica were detected in Ethiopia. *Salmonella* Kentucky of the European serovar was also isolated from poultry and human in Ethiopia (Betelhem *et al*., 2020).

In Ethiopia, the prevalence of *Salmonella* serotypes affecting poultry industries varies. Some research reports showed the prevalence to be as high as 15.12% (Abda *et al*, 2021). According to Belachew, 2021, Salmonella was isolated from 24.30% of the samples tested (Table 3). A study conducted in central Ethiopia reported that 14.6% of poultry farms in central Ethiopia were positive for Salmonella (Betelhem *et al.*, 2020) while studies conducted on retail raw chicken products reported a 17.9% prevalence of Salmonella (Eguale, 2018). Eguale, 2018 also reported that *Salmonella* Saintpaul was the dominant serotype isolated while, *S.* Typhimurium, *S.* Kentucky, and *S.* Haifa were also detected. A study conducted on eggs to examine Salmonella showed 11.5% positivity, of which 10.5%

positivity was found from eggs collected from poultry farms while 3.0% were from open markets (Mintesnot *et al*, 2011).

Table 3. Prevalence of Salmonella in Chickens (*S.* Gallinarum, *S.* Pullorum*; S.* Enteritidis, *S.* Typhimurium*; S.* Haifa, *S.* Anatum, *S.* Give, *S.* Kentucky, and *S.* Saintpaul)

Total sample	Prevalence (%)	Location	Reference
539	24.3	Chico meat broiler farm (Bishoftu)	Belachew *et al.,* 2021
302	9.27	Kafa zone, southwest	Abda *et al.,* 2021
836	2.9	poultry farms in Adama and Modjo towns	Betelhem *et al., 2020*
205	19*	West Shoa Zone (Ambo, Holeta, Guder, Ijaji, and Dire Inchini districts)	Edilu *et al.,* 2020
384	14.6	Debre Zeit and Modjo towns	Destaw *et al.,* 2020
549	4.7	Addis Ababa and its surrounding districts (Ada'a, Sebeta, Barake)	Eguale, 2018
196	6.7*	Debre Zeit	Yizengaw, 2015
150	9.33	intensively managed chickens from Cheffe, Jarso, and Horro eco-types of local and an exotic breed, Fayuomi (in DZ)	Medina *et al*., 2013
770	32.8	Mekelle poultry multiplication center and backyard poultry around Mekelle	Netsanet *et al.,* 2012
400*	11.5	Kombolcha poultry multiplication & breeding	Mintesnot *et al.,* 2011
-	35.9		Berihun, 2007
-	64.2	in Central Ethiopia	Ashenafi *et al*., 2003
-	1.5	East Showa	Dereje, 2002

* 6.7% molecular test results 19% culture-based prevalence *400 egg sample

All species of birds are susceptible to Salmonella infection. However, the outcome of infection depends on a variety of factors, including age, host species susceptibility/breed, flock size, and bacterial virulence. *Salmonella* Pullorum and *Salmonella* Gallinarum can result in high mortality in birds of all ages. Although pullorum disease affects birds at any age, mortality rates are higher in young animals. The ability to survive infection increases with age while mortality is greatest in newly hatched chicks (Netsanet *et al.*, 2012).

Mortality in the acute form ranges from 20 to 80 percent and in the chronic form is around 5 percent (Netsanet *et al*., 2012).

Poultry is also host to the zoonotic forms of salmonella, *S.* Typhimurium and *S.* Enteritidis. The non-host-specific *Salmonella* serovars usually infect a wider range of hosts and cause disease in humans as well. Poultry and poultry products are the major sources of human infection with non-typhoidal Salmonella (Netsanet *et al*., 2012). It has been shown that some of the most commonly detected serovars in chickens in a given geographic area are also among the top serovars associated with human infections indicating the role of Salmonella colonization of poultry farms to public health (Eguale, 2018). The existence in humans of *Salmonella* serovars which are most prevalent in poultry, suggests a possible epidemiologic connection between poultry as a reservoir of *Salmonella* and human infection (Netsanet *et al*., 2012).

The situation in Ethiopia is more liable to aggravate the current epidemiological connection between poultry diseases and human infection with zoonotic pathogens. Most of the small-scale semi-intensive poultry farms in urban and peri-urban areas of Ethiopia are located near human residential areas suggesting the possibility of transmission of potential pathogens of poultry to humans. Some of the smallholder poultry producers keep chickens in the same compound where they live. This could expose the family members to the transmission of zoonotic pathogens (Betelhem *et al*., 2020).

2.3.3. Public Health Significance of Bacterial Poultry Diseases

There is an extensive exchange of bacteria among confined food animals, wild animals, humans, and the environment. Studies on bacterial pathogens provide strong evidence for the bidirectional transfers of pathogens among poultry grown in confined operations, wild birds, and human populations. Some of the bacteria that are associated with chickens and have the potential to be transmitted to humans are *Salmonella* Enteritidis, *Staphylococcus aureus*, *Campylobacter jejuni*, and *Listeria monocytogenes* (Lm) (Jay *et al.*, 2008). Foodborne zoonoses like *Salmonella*, *E. co*li, and *Campylobacter* (which lives in chickens

but causes little disease) can be transmitted to people via chicken droppings or can contaminate chicken meat and, in the case of Salmonella, eggs. These zoonotic diseases may cause diarrhea, especially in children, and can cause invasive disease, especially in people with HIV or malaria where infection can be fatal. Since their immune systems are not yet fully developed, children are more likely to get sick from germs commonly associated with poultry, such as *Salmonella*, *Campylobacter*, and *E. coli* (Marisa, 2009).

Foodborne diseases are among the most widespread global public health problems of recent times, and their implication for health and the economy is increasingly recognized. In this regard, among bacterial zoonotic agents, non-typhoid salmonella groups and campylobacter are of higher priority. They are among the most important food safety hazards. These bacteria account for more than 90 percent of all reported cases of bacteria-related food poisonings worldwide. Most of these cases are related to the consumption of poultry and poultry products, but all domestic livestock are potential reservoirs of infection (Yohans *et al*., 2019).

Salmonella and Campylobacter are common public health hazards that are potentially associated with chicken contact. These bacteria are carried by healthy chickens and are communicable to people through direct contact, exposure to manure, or consumption of undercooked chicken and eggs. However; some of the zoonotic agents may not cause clinical illness in humans, particularly among those who have solid immunity. The host-adapted salmonella, *Salmonella* Gallinarum, and *Salmonella* Pullorum rarely cause clinical cases in people (Marisa, 2009).

2.3.3.1. Campylobacteriosis

Campylobacter infections are among the most important food safety hazards. They have raised potential public health concerns and are of higher priority and can be transmitted to people via chicken droppings or contaminated chicken meat (Yohans *et al*., 2019). Campylobacteriosis is becoming the most frequently reported zoonosis. Transmission of Campylobacteriosis occurs *via* direct contact with feces or by food cross-contamination

due to raw or undercooked meat and other contaminated foods. Different routes by which humans are exposed to Campylobacter include ingestion of contaminated food and water, direct contact with infected animals or carcasses, from environments contaminated with animal feces or sewage, or direct person-to-person transmission. During processing, especially if the intestinal tract is ruptured and the contents are moved to the skin, prompting further pollution of the carcass. Likewise, contact of eggs with fecal material results in egg contamination and the transmission of the bacteria to the inside of the egg, thus initiating the disease after the consumption of eggs (Ghoneim *et al.,* 2021).

Campylobacteriosis is the main bacterial foodborne disease in humans. It causes Campylobacteriosis of considerable public health significance and its presence in chickens is a significant risk for zoonotic infection. In countries with a highly developed intensive poultry industry, there is considerable epidemiological evidence that chickens are the main source of human Campylobacter infection. In developing countries, far less is known about the epidemiology of Campylobacter, but it has been found at high levels in retail poultry meat (Brena *et al.,* 2016). Due to this, there is a possible risk of transmission of even the highly virulent *C. jejuni* as a foodborne pathogen from both broiler and layer chickens to humans (Ghoneim *et al.,* 2021).

Because poultry has a higher prevalence of Campylobacter in their natural microbiota, they are the main foods involved concerning public health. Foodborne Campylobacter infections occur as a result of the consumption of undercooked or raw poultry, liver, or grilled chicken meat. This may be due to the thermophilic properties of Campylobacter species, especially *C. jejuni*, which favors poultry hosts due to their high body temperatures (Ghoneim *et al.,* 2021). It has been widely accepted that improper handling and consumption of contaminated food (notably poultry meat) accounts for the majority of human cases. In humans, *C. jejuni* and *C. coli* are the main offenders of Campylobacteriosis, a very widely recognized enteric illness that can be transmitted to humans through the consumption of undercooked meat, especially poultry, contaminated water and egg, and contact with farm animals such as poultry and livestock (Nikki *et al*., 2019).

Campylobacteriosis in humans is characterized by watery and/or bloody diarrhea, abdominal pain, cramps, fever, malaise, and vomiting. This is especially dangerous for young children who are more prone to dehydration and loss of nutrients, such as sodium and protein, as a consequence of the diarrheal illness (Nikki *et al*., 2019). Thus, *Campylobacter* species are considered the second driving etiology of pediatric diarrhea (Ghoneim *et al.*, 2021).

In Ethiopia, human Campylobacteriosis has been reported at different prevalences. The meta-analysis conducted by Zenebe *et al*., 2020 had shown that the prevalence of Campylobacter in humans was 9% based on the pooled prevalence of *Campylobacter* species from different sources. Studies on the prevalence of Campylobacter in humans have identified a range of 13.8–50.0% (Chala *et al*., 2021). Desalegne and Adane, 2010 also reported an overall prevalence of thermophilic campylobacters being 8% in humans.

2.3.3.2. Salmonella

Salmonella is one of the common bacterial pathogens transmitted from poultry and poultry products to humans (Betelhem *et al*., 2020). Birds can carry germs like Salmonella while looking healthy and clean. Salmonella germs can spread between birds, and to people. Salmonella is spread by the fecal-oral route and can be transmitted by food and water, by direct animal contact, and rarely from person-to-person. An estimated 94% of salmonellosis is transmitted by food. Humans usually become infected by eating foods contaminated with feces from an infected animal. Foods of animal origin, especially poultry and poultry products, are often involved in sporadic cases and outbreaks of human salmonellosis (Abunna *et al*., 2016).

Consumption of contaminated poultry and poultry products represents a common source of nontyphoidal Salmonella infection. Consumption of contaminated animal-derived food products and contact with animals are ways through which nontyphoidal Salmonella is mainly acquired. Touching the mouth with unwashed hands after touching wild birds, bird

feeders, or bird baths that have contact with wild birds will let someone get infected with Salmonella (Betelhem *et al*., 2020).

Salmonella is one of the most prevalent zoonotic pathogens in both developed and developing countries. In Ethiopia, the detection of salmonellosis with high overall prevalence, a higher proportion of those with likely zoonotic potential and multi-drug resistant isolates, indicated that salmonellosis could be an emerging poultry and public health problem (Abunna *et al*., 2016). Tadesse, 2014 conducted a systematic review and meta-analysis on the prevalence of human Salmonellosis in Ethiopia and reported that the pooled prevalence estimates of Salmonella in stool samples of diarrheic children, diarrheic adults, and carriers were 8.72%, 5.68%, and 1.08% respectively while the non-typhi isolates accounted for 57.9% of the isolates from patients. Betelhem *et al*., 2020 also reported that 2.8% of stool samples of humans in contact with poultry tested positive for Salmonella.

Among the infectious diseases, poultry salmonellosis is one of the main problems of economic concern for direct and indirect losses to all phases of the poultry industry from production to marketing (Netsanet, *et al*., 2012). Particularly, Fowl typhoid and pullorum disease are mentioned to cause heavy losses (Medina et al., 2013). Animals that survive may become carriers, may not meet expected animal production parameters, and may produce contaminated eggs. Artificial incubation of eggs was highly influenced by the occurrence of the disease because it led to high mortality and culling rates among chicks (Netsanet *et al*., 2012). Although the principal current economic significance of *S.* Pullorum in developed nations is the cost of testing programs, reminders of the potential for catastrophic losses have been provided by the occasional appearance of Pullorum disease in commercial flocks.

Poultry salmonellosis causes great economic losses, but no data are available on the possible economic losses they incur, however, scattered documents revealed that it causes high mortality rates which can reach up to 100%, a decrease in production (eggs and chicks), condemnation of affected carcass and viscera at abattoirs and cost of medication

both in humans and animals. Direct health costs include (e.g. hospitalization, consulting a physician, and laboratory testing) as well as the costs of lost labor (e.g. loss of production per day away from work) in relation to a case of salmonellosis. Antibacterial treatment helps to reduce mortality but treated chicken remains carriers (Netsanet *et al*., 2012).

2.4. Major Viral Poultry Diseases in Ethiopia

Viral diseases are common among different poultry farms in Ethiopia (Mulisa *et al.*, 2014). The high-priority viral poultry diseases in Ethiopia include Newcastle disease (ND), Infectious bursal disease (IBD), Marek's disease (MD), Infectious bronchitis (IB), Infectious laryngotracheitis (ILT), and Avian influenza (AI) in order of their report rate and the number of publications on the diseases (ND = 17, IBD = 10, MD = 2 and Infectious bronchitis = 1) (Yohannes *et al*., 2019). Since reporting is a dynamic process and research and publication of the research works are ongoing activities, the figures and the reported diseases can vary. Recently there were reports of IB, ILT, and aMPV in Ethiopia in addition to the above-mentioned diseases (Hutton *et al*., 2017; Tesfaye *et al.*, 2019; Asamenew *et al*., 2019; Roba *et al*., 2020). There were also reports of the low pathogenicity AI (LPAI) in Ethiopia which was last reported in 2006 while so far there was no confirmed case of the high pathogenicity AI (HPAI), the H5N1 strain, in chickens in Ethiopia (Hutton *et al*., 2017). But owing to the structure of the Ethiopian poultry sector that has a bearing on the risks for the introduction of disease into the country and new farms, different researchers have pointed out the existence of the risk of introduction and spread of HPAI that has put Ethiopia at risk of HPAI outbreak (Dawit *et al*., 2008).

Among the mentioned viral poultry diseases, ND, IB, AI, ILT, and MD cause respiratory disease while IBD and MD cause immunosuppression, predisposing poultry to different health problems and making them non-responsive to vaccine interventions for prevention or reduction of infections. Particularly, IBD that affects the bursa of Fabricious destroys immature B-lymphocytes within the bursa of Fabricious and provoked immunosuppression, and causes considerable morbidity and mortality either directly in an acute form or as a consequence of secondary infections (Arianne *et al.*, 2018).

Outbreaks of ND have been reported at different times in commercial poultry farms in Ethiopia, despite the routine vaccination exercised, affecting more than half-million chickens and killing 20% of them (Dawit *et al*., 2008). The mortality rate due to ND remains high (Alemu *et al*., 2008). In Ethiopia, the Newcastle disease virus (NDV) is identified as a major killer, largely contributing to economic losses for the poultry sector (Habte *et al*., 2017). In flocks with no history of vaccination, some authors reported seroprevalence values for NDV ranging between 5–64.1% (Hailu, 2010; Chaka *et al*., 2012). These studies revealed that the majority of the Newcastle disease virus strain circulating in village chickens in Ethiopia was the virulent strain of the class II virus that is grouped in the sub-genotype VIf (Mulisa *et al*., 2014).

Next to ND, other infectious poultry diseases such as IBD/Gumboro and Marek's diseases have occurred in the country most likely related to the importation of live vaccines of the respective diseases (Dawit *et al*., 2008). The recently reported diseases like IB, ILT, and aMPV were also common in Ethiopia (Hutton *et al*., 2017; Tesfaye *et al.*, 2019; Asamenew *et al*., 2019; Roba *et al*., 2020). Among causative agents of these viral diseases, the Infectious bursal disease virus (IBDV) is a pathogen that usually affects young chickens and weakens their immune system, predisposing the birds to vaccination failure and opportunistic pathogens (Rautenschlein and Alkie, 2016).

IBDV is an emerging disease in Ethiopia, and it was detected in 2002 for the first time (Zeleke *et al*., 2005). Its circulation seems to be worsened by the adoption of exotic breeds of chickens, which are considered less resistant than indigenous breeds (Mekuriaw *et al*., 2017; Hinsemu *et al*., 2018). From the few commercial poultry farms situated in the central part of Ethiopia, in which the disease was first reported, now IBDV has widely spread to other parts of the country (Berhanu *et al*., 2018). Now, the IBDV seroprevalence in the country reaches 83.1% (Degefu *et al*., 2010; Jenbreie *et al*., 2012; Zeryehun and Fekadu, 2012). The mortality rates due to IBDV in Ethiopian chickens were also reported to reach 50% (Zeleke *et al*., 2005). Studies have revealed that it is the very virulent IBDV (vvIBDV) strains that are circulating in Ethiopia (Negash *et al*., 2012; Shegu *et al*., 2020). Generally, the research reports have shown that the Ethiopian IBDVs represented two genetic

lineages: the very virulent (vv) IBDVs and variants of the classical attenuated vaccine strain (D78) (Jenberie *et al*., 2014), and also other vaccine strains: B2K, LC75, EXTREM and Winterfield-2512 (Mekuriaw *et al*., 2017; Shegu *et al*., 2020).

Despite regular vaccination practices, IBDV is still found in Ethiopia involving both commercial and backyard poultry (Mekibib *et al*., 2019). Low biosecurity standards contribute to the spread of IBDV with risk factors such as visitors from different poultry houses, and uncontrolled movement among different poultry houses of workers of poultry farms and vendor vehicles aggravating the transmission of the virus (Tulu, 2019).

Infectious bronchitis virus (IBV) and avian metapneumovirus (aMPV) were also recently found in Ethiopia, but at lower occurrence levels (Hutton *et al*., 2017; Tegegne *et al*., 2020). Avian infectious bronchitis (IB) is an important disease of poultry that affects the respiratory tract, gut, kidney, and urogenital and reproductive systems of chickens (Ignjatovi´c and Sapats, 2020). Few studies conducted in different parts of Ethiopia have reported IBV seroprevalence to be high on both commercial and backyard chicken farms. Four serotypes of infectious bronchitis virus were identified from backyard and commercial farms in Ethiopia, namely M41 (GI-1), D-274 (GI-12), 793B (GI-13), and QX (GI-19) (Tesfaye *et al*., 2019). The detection of a strain belonging to the 793B genotype (GI-13) (Hutton *et al*., 2017), together with another study reporting the detection of 793B-like (GI-13) strains in distantly spaced backyard flocks (Tegegne *et al*., 2020) suggest a relevant viral circulation in Ethiopia. In these studies (Hutton *et al*., 2017; Tegegne *et al*., 2020), the authors also reported aMPV subtype B, both in the backyard and intensively raised flocks, with respiratory signs.

Avian metapneumovirus is a respiratory pathogen whose importance is growing in poultry. Vaccination for aMPV is not commonly adopted in chickens, especially in Ethiopia, where it is currently unavailable (Hutton *et al*., 2017). Thus, the control of this agent has mainly relied on biosecurity. The introduction of aMPV has been tentatively linked to the importation of birds (Tegegne *et al*., 2020). Mortality rates are usually low, except for cases

of secondary infection that can result in severe forms, in particular with *Escherichia coli* (Suarez *et al*., 2020).

2.4.1. Newcastle disease (ND)

Newcastle disease is an infection of birds that is highly infectious and causes more losses than any other disease in the tropics (Matawork, 2016). The NCD produced in poultry varies from extremely pathogenic to inapparent, depending on the strain of the virus and the host infected.

Newcastle disease (ND) is enzootic in some areas of the world and a constant threat to most birds reared domestically (Alexander, 2000). Studies revealed that the majority of the virus strains circulating in the village chickens in Ethiopia are virulent strains with multiple basic amino acids at the cleavage site of F protein 112RRQKR116*F117. The isolates so far identified in Ethiopia are grouped in the new sub-genotype VIf of class II viruses based on phylogenetic analysis of the 260 fragments of the fusion gene of the viruses (Mulisa *et al.*, 2014). Based on similarity analysis by complete coding region of the F gene of recent isolates with previously isolated NDV in Ethiopia, they are found similar, suggesting the persistence of identical virus with the above sub-genotype (VIf of class II viruses) in poultry in the country. It was suggested that this could be associated with weak biosecurity practices and the exposing marketing channel system in Ethiopia. The existence of co-circulation of a different genotype, genotype VII was also found in Ethiopia (Mulisa *et al.*, 2014) (Table 4).

Table 4. Seroprevalence of Newcastle disease in Ethiopia

N	% positive	Location	Reference
44	38.63*	Central Ethiopia (sick & dead chickens)	Worku *et al*., 2022
84**	15.48*	Rift Valley (unvaccinated village chickens)	Ashenafi *et al.,* 2021
300	26.7*	Market and village chicken in East Shewa zone	Yemsrach *et al., 2016*
384	27.86	Sinana and Agarfa	Minda *et al*., 2016
155	11.6	village chickens in Bishoftu, Tikur wuha, and Ziway districts	Terefe *et al*., 2016
521	28.6	village chicken in the Lume district	Desalegn, 2015
146**	30.1*	live poultry markets in Addis Ababa	Mulisa *et al*., 2014
1056	0.007	village chicken in Horro and Jarso	Bettridge *et al., 2014*
-	6	Eastern Shewa zone	Chaka *et al*., 2012
-	29.6	Bahir Dar	Hailu *et al., 2009*
-	21.70	Farta	" "
-	100	Andasa poultry farm	Solomon & Abebe, 2007
-	11	Southern Ethiopia	Regasa *et al., 2007*
-	19.78	Village Chickens in Southern and Rift Valley areas	Zeleke *et al*., 2005a
283	93.30	Debre Zeit	Zeleke *et al*., 2005b
180	32.22	Debre Berhan, Sebeta, Nazareth	Tadesse *et al*., 2005
-	43.68	Local scavenging chickens in Central Ethiopia	Ashenafi, 2000

N = total sample *molecular-based prevalence ** sample pool

Newcastle disease (ND) is highly infectious and is one of the major causes of economic loss to poultry production in Ethiopia (Dawit *et al*., 2008). It is identified as the major killer contributing a large share to the loss of the poultry sector. Newcastle disease often wipes out the whole flock when it strikes. It spreads rapidly through the flock and could cause mortality that reaches up to 100% (Matawork, 2016). The incidence of Newcastle disease is widespread during the rainy season. It was found that poultry production drops by 50% during the rainy season (Dawit *et al*., 2008) (Table 5).

Table 5. Mortality rates of Chickens due to Newcastle disease in Ethiopia

Mortality (%)	Location	Reference
62.1	village chicken in the Lume district	Desalegn, 2015
38.9	Bahir Dar	Hailu *et al*., 2009
17.40	Farta	" "
49.89	Debre Zeit (overall mortality)	Zeleke et al., 2005b
25.08	Layer morality	" "
56.09	Broiler mortality	" "
43.7	Central highlands of Ethiopia	Ashenafi, 2000

2.4.2. Infectious bursal disease/Gumboro (IBD)

Infectious bursal disease (IBD) is an emerging disease of chicken in Ethiopia associated with the introduction of exotic breeds of chicken into the country. The disease has been speculated to be introduced concurrently with the increased number of commercial state and private poultry farms flourishing in Ethiopia (Mekuriaw *et al*., 2017). From the few commercial poultry farms situated in the central part of Ethiopia in which the disease was first reported, IBD was widely spreading to other parts of the country (Berhanu *et al*., 2018). Now, Infectious Bursal Disease (IBD) is the most important threat to poultry production in Ethiopia and is widely distributed in all regions in commercial farms, poultry multiplication centers, and backyard chickens. Studies indicated the presence of the disease in many parts of Ethiopia and that IBD incidences are increasing at an alarming rate all over the country where commercial poultry production is intensified and also in the backyard chickens. The documented results indicated that IBD is a threat to both backyard chickens and commercial chickens. The situation of the disease both at small-scale commercial flocks and backyard poultry farms also indicated the disease is widely distributed in the country.

The causative virus weakens the immune system leaving birds highly susceptible to secondary infections as well (Arianne *et al.*, 2018). The sequellae of IBDV infections such as the severity of clinical signs, organ lesions, and immunosuppression correlates with the status of immunity, age, and genetic background of affected chickens and with the

virulence of the infecting virus strain (Terefe, 2018; Mekuriaw *et al*., 2017). IBD virus remains infectious for a very long time and has resistance to commonly used disinfectants. The virus is quite resistant to physical and chemical agents, resistant to pH conditions of 2–11, but it is inactivated at pH 12. The virus has been shown to remain infectious for 122 days in a chicken house, and 52 days in feed, water, and feces. Due to this ability of stability and hardiness, it persists in poultry premises even after thorough cleaning and disinfection (Mekuriaw et al., 2017).

The segmented double-stranded RNA genome of the Infectious bursal disease virus (IBDV) is responsible for the virus being prone to genetic variation. As a result of this, there are IBDV isolates with different genotypic and phenotypic diversity. The consequences of immunosuppression associated with IBDV are vaccination failure and susceptibility of chickens to opportunistic pathogens (Alkie and Rautenschlein, 2015). Due to this, despite regular vaccination practices, IBD is still found in Ethiopia involving both commercial and backyard poultry production systems (Berhanu *et al*., 2018). Most of the researchers in Ethiopia performed a serological survey in different parts of the country. These studies indicated that the disease in commercial farms and multiplication centers occurs at an average outbreak rate of 3-4 farms per year (Terefe, 2018).

The genotyping and molecular characterization of IBDV in Ethiopia based on studies conducted at different times showed that it is the vvIBDV that is circulating in the country (Negash *et al*., 2012; Jenberie *et al*., 2014; Shegu *et al*., 2020). Besides the very virulent (vv) IBDVs, variants of the classical attenuated vaccine strain (D78) (Jenberie *et al*., 2014), and other vaccine strains, namely B2K, LC75, EXTREM and Winterfield-2512 (Mekuriaw *et al*., 2017; Shegu *et al*., 2020) were identified in Ethiopia (Table 7).

Table 6. Seroprevalence of IBDV in Chickens in Ethiopia

Total sample	Overall Prevalence (%)	Site	Reference/Author
-	92	in backyard chickens	Wagari, 2021
706	77.48	Diff. sites	Dereje, 2019
-	75	West Gojam	Samson, 2017
-	72	Gondar	Samson, 2017
-	38.3	Sebeta	Tesfaye *et al*., 2016
384	45.05	Mekelle town	Zegeye *et al., 2015*
521	20.7	Lume district	Desalegn, 2015
552	83	Eastern Ethiopia (Adama, Modjo, Wenji)	Tadesse and Jenbere, 2014
1056	3.6	Horro and Jarso region	Bettridge *et al*., 2014
276	82.2	Back yard in Bishoftu	Zeryehun and Fekadu, 2012
2,597	83.1	Mekele, Gondar	Jenberie *et al*., 2012
-	92	backyard chickens	Chaka *et al.*; Jenbreie *et al., 2012*
-	73.50	Gondar and west Gojjam	Kassa and Molla, 2012
-	89.8	Woliso	Degefu *et al*., 2010
-	70.7	Ambo	Degefu *et al*., 2010
-	40.8	Holeta	Degefu *et al*., 2010
-	29.4	Bahir Dar	Hailu *et al*., 2010
-	21.7	Farta	Hailu *et al*., 2010
775	27.8	Bahir Dar	Hailu *et al*., 2009
775	77.73	Farta	Hailu *et al*., 2009
-	76.64	Southwest showa	Hailu *et al., 2009*
-	51.1	Bahir Dar	Mazengia, 2009
-	100	Andasa poultry farm	Solomon and Abebe, 2007
-	61	Addis Ababa & Adami Tulu	Nigussie, 2007
-	93.3	different farms	Zeleke *et al., 2005b*

Mortality

The infectious bursal disease causes considerable morbidity and mortality thus resulting in economic losses in an acute form or as a consequence of severe immunosuppression. Highly virulent IBDV can cause high mortality in unprotected flocks. The mortality rates of the disease in Ethiopian chickens were reported to be as small as 10.4% in Commercial farms in Bishoftu, Asella, Kombolcha, Sululta, Addis Ababa (Dereje, 2019) and as high as 98.56% in Bahir Dar (Hailu, 2009) (Table 6).

Table 7. Mortality in Chicken due to IBD cases in Ethiopia

Mortality (%)	Site and Breed	Reference
66.93	Western Oromia	Abdeta *et al*., 2022
79.43	Waliso district in apparently healthy chicken	Bedasa *et al*., 2021
47.9 (10.4-80)	Commercial farms in Bishoftu, Asella, Kombolcha, Sululta, Addis Ababa	Dereje, 2019
50-72	Chicken	Terefe, 2018
25-75	Exotic and cross chickens	Mekuriaw *et al*., 2017
38.9	Bahir Dar	Hailu *et al*., 2010
17.4	Farta	``
98.56	Bahir Dar	Hailu, 2009
72	Andasa poultry farm (young mortality)	Solomon & Abebe, 2007
7	Andasa poultry farm (adult mortality	``
49.89	Debre Zeit (overall mortality)	Zeleke *et al, 2005b.*
56.09	Broiler (mortality)	Zeleke et al., 2005b
25.08	Layer (morality)	Zeleke et al., 2005b
45-50	Privately owned commercial poultry farm	Zeleke *et al., 2003*

Specific pathogen-free (SPF) chickens infected with vvIBDV develop an earlier onset of mortality and more severe bursal lesions compared to broiler chickens with maternal antibody (MAB) and vaccinated chickens (Terefe, 2018).

2.4.3. Infectious bronchitis disease (IB)

Avian infectious bronchitis is a highly contagious respiratory disease in poultry. It affects the respiratory tract, gut, kidney, urogenital and reproductive systems of chickens causing considerable losses due to death, egg drop, and reduced meat production (Arianne *et al.,* 2018).

Little information is available on infectious bronchitis in chickens in Ethiopia. A study conducted in commercial and backyard farms in Oromia and the Southern Nations Nationalities and People's (SNNP) regional States found that out of 711 chickens tested, 70.60% were seropositive to IBV. The prevalence was 68.75% and 74.88% in chickens raised in commercial settings and backyard systems, respectively. Four serotypes of infectious bronchitis virus namely M41, D-274, 793B, and Qx were identified from the

unvaccinated backyard and commercial farms. The four serotypes of IBV tested are widespread in many backyard and commercial farms in Ethiopia. The seroprevalence observed is high in both commercial and backyard chicken farms (Tesfaye *et al*., 2019).

For the first time in Ethiopia, the detection of a variant infectious bronchitis virus (793B genotype) was reported in a chicken breeder farm in Debre Zeit that is run by the Ethiopian Institute of Agricultural Research (EIAR)). The seroprevalence rate of IBV was found to be 94.5% (Hutton *et al*., 2017) (Table 8).

Table 6. Seroprevalence of IBV in Chickens in Ethiopia

Total sample	Prevalence (%)	Location	Reference
354	97.46	Bishoftu and Holeta	Jirata *et al*., 2022
768	23.96	Northwest Ethiopia	Birhan *et al*., 2021
500	6*	Jimma zone	Tegegne *et al*., 2020
711	70.60	Oromia and SNNP (Overall)	Tesfaye *et al*., 2019
	68.75	Commercial farms	`` ``
	74.88	Backyard farms	`` ``
834	94.5	Chicken breeder farm in Debre Zeit	Hutton *et al*., 2017

*molecular test result

2.4.4. Avian metapneumovirus (aMPV)

Avian metapneumovirus causes a variety of disease syndromes in birds, depending on the bird species and virus type (A, B, C, or D). Type A and B cause acute respiratory tract infection in turkeys, or swollen head syndrome in broilers. Reliant to strain, temperature, and time of exposure, aMPV is capable of resisting a pH from 3 to 9. The virus is inactivated at 56 °C (133 °F) after 30 minutes and is destroyed by commonly used disinfectants. For the first time in Ethiopia, the detection of avian metapneumovirus subtype B in poultry was reported with a seroprevalence of 21.9% (Hutton *et al*., 2017).

In general, even if attempts were made to control the prevalent viral poultry diseases in Ethiopia, their distribution is still prevailing in the country. As referred from the continuous

reports through the published research papers on the diseases, it can be concluded that all the significant viral poultry diseases in Ethiopia have remained a problem to the poultry sector as well as to the human population imposing public health and economic significance.

2.4.5. Public Health Significance of Viral Poultry Diseases

Some poultry diseases have public health significance. These diseases can spread via occupational contact with infected chickens or the consumption of chicken meat and eggs (Teferi, 2016). Even if poultry meat and eggs provide nutritionally beneficial food containing protein of high quality, always there are risks of acquiring infection associated with the consumption of these products, particularly raw products (Tessari *et al.*, 2011). Human contact with live poultry, both in small household farms and in industrial operations, is a clear risk factor for exposure to avian commensals and other pathogenic poultry diseases particularly viral diseases that can infect humans (Leibler *et al*., 2008).

2.4.5.1. Newcastle Disease - Public Health Impacts

Humans can be infected by NDV that have usually resulted from direct contact with the virus, infected birds, or carcasses of diseased birds. Human-to-human spread has never been reported, although spread by contagion is theoretically possible. The types of people known to have been infected with NDV include laboratory workers (usually as a result of accidental splashing of infective material into the eye), veterinarians in diagnostic laboratories (presumably as a result of contact with infective material during post-mortem examinations), workers in broiler processing plants and vaccination crews, especially when live vaccines are administrated as aerosols or fine dust. Newcastle disease viruses that infect humans usually cause transient conjunctivitis. The most frequently reported and best-substantiated clinical signs in human infections have been eye infections, usually consisting of unilateral or bilateral reddening, excessive lachrymation, edema of the eyelids, conjunctivitis, and sub-conjunctival hemorrhage. Reports of other clinical symptoms in humans infected with NDV are less well substantiated but suggest that a more

generalized infection may sometimes occur, resulting in chills, headaches, and fever, with or without conjunctivitis. Both vaccinal strains and strains virulent to poultry may infect and cause clinical signs in humans (Alexander, 2000).

The economic loss to NDV is related not only to high mortality and production losses it causes but also results in economic losses by trade restrictions. NDV is considered an important constraint for poultry products throughout the world especially in developing countries. The disease leads to high mortality and morbidity of chickens, high medication costs, and loss in production and market (Asfaw *et al*., 2021).

2.4.5.2. Infectious Bursal Disease - Public Health Impacts

IBD has not been reported to have any zoonotic potential (Dereje, 2019). Though IBD has no direct public health significance, its immunosuppressive nature could cause public health-significant disease agents to flare up and cause infection in humans. That is, it may pose a significant impact on public health by favoring the development of several poultry infections of zoonotic importance like Salmonellosis, Campylobacter, and Avian influenza. Due to its immunosuppressive effect, affected flocks show increased susceptibility to infection with opportunistic pathogens, often leading to chronic disease situations and suboptimal vaccine responses (Berhanu *et al*., 2018). IBDV is economically important to the poultry industry worldwide due to increased susceptibility to other diseases and negative interference with effective vaccination.

The economic impact of IBD in fowl is serious and influenced by the strain of the virus, susceptibility, and breed of the flock; intercurrent primary and secondary pathogens, and environmental and managemental factors. Clinical IBDV leads to direct losses due to high mortality. Besides, condemnation of carcasses due to skeletal muscle, thigh, and pectoral muscle hemorrhages can be an important cause of economic losses. Indirect losses in Gumboro disease arise due to the severe immunosuppression of broilers and egg-laying hens and their increased predisposition for other diseases and vaccination failure. Thereby, as a consequence, they result in delayed growth, reduced weight gain, greater food

conversion, longer fattening, lesser production values, increased mortality, and lower quality of products (Mekuriaw *et al*., 2017).

Earlier, the strains of the IBD virus were of low virulence that causes less than 2% mortality, and vaccination was able to satisfactorily control the disease. However, the recently occurred vvIBDV has led to vaccination failures and increased mortality and morbidity. This occurrence of vvIBDVs has increased the economic importance of the disease (Terefe, 2018; Mekuriaw *et al*., 2017).

The presence of the disease may also limit opportunities in the marketplace, either locally or internationally, and hinder the adoption of improved technologies, be the improved breeds, better management systems, or more efficient processing and marketing methodologies. There would be further loss of income for an extended period because of the stamping-out policy. The disruption to the flow of products and decreased production may cause job losses on farms and in service and associated industries, depending on the time it takes to bring the outbreak under control. Even a small outbreak would result in the dislocation of the industry and its normal marketing patterns. An uncontrolled outbreak would markedly increase production costs because of the impact of the disease and the need for continuing control measures (Terefe, 2018; Mekuriaw *et al*., 2017).

2.4.5.3. Infectious Bronchitis - Public Health Impacts

No risks to human health are suspected or have been demonstrated to arise from IBV. However, neutralizing antibodies have been detected in people working with commercial chicken flocks, but the significance of this remains unknown. No evidence exists to suggest that humans act as a reservoir for active replication of IBV and no evidence has been found for its transmission from human to human, or human to animal. Humans can only transmit IBV to chicks by mechanical means (Ignjatovic and Sapats, 2000).

3. MATERIALS AND METHODS

3.1. Study Area

This study was carried out in Bishoftu and Mojo, which are located in the Oromia region, Central Ethiopia, in the proximity of Addis Ababa (Fig. 1). Bishoftu is located 45 Km southeast of Addis Ababa. The area is located at 9^{o}N latitude and 40^{o}E longitude at an altitude of 1850 meters above sea level. The temperature in Bishoftu varies so little throughout the year that it is not entirely meaningful to discuss hot and cold seasons, as do in most parts of Ethiopia. Over the year, the temperature of Bishoftu typically varies from 12°C to 27°C and is rarely below 9°C or above 30°C. The area has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September) (NMSA, 2010). Bishoftu is a town where several commercial farms (>50) of different scales, hatcheries, and breeding farms are found, and from which chickens of various ages are distributed to different parts of the country. The total poultry population of East Shewa to which Bishoftu belongs was estimated to be 859,616 (CSA, 2021). One of the private large-scale commercial poultry farms in Bishoftu, i.e. Alema, has modern poultry abattoirs. This abattoir slaughters broiler chicken supplied from large commercial and small-scale poultry farms and deliver chicken meat for the local market mainly supermarkets and hotels in Addis Ababa and its surrounding.

Mojo is a town located in the intermediate highland agroecology in central Ethiopia. The distance from Mojo to Addis Ababa is 64 kilometers. Mojo is located at 8° N latitude and 39° E longitude at an altitude 1797 m above sea level. In Mojo, the wet season is mostly cloudy, the dry season is partly cloudy, and it is warm year-round. Over the year, the temperature typically varies from 13°C to 28°C and is rarely below 10°C or above 31°C. The average annual temperature in Mojo is 20.1 °C/68.1 °F. The rainfall here is around 863 mm/34.0 inches per year. Intensive poultry production system is practiced in the town and the nearby areas. Intensive poultry production was relatively concentrated in the town. The total poultry population of East Shewa to which Mojo belongs was estimated to be 859,616 (CSA, 2021).

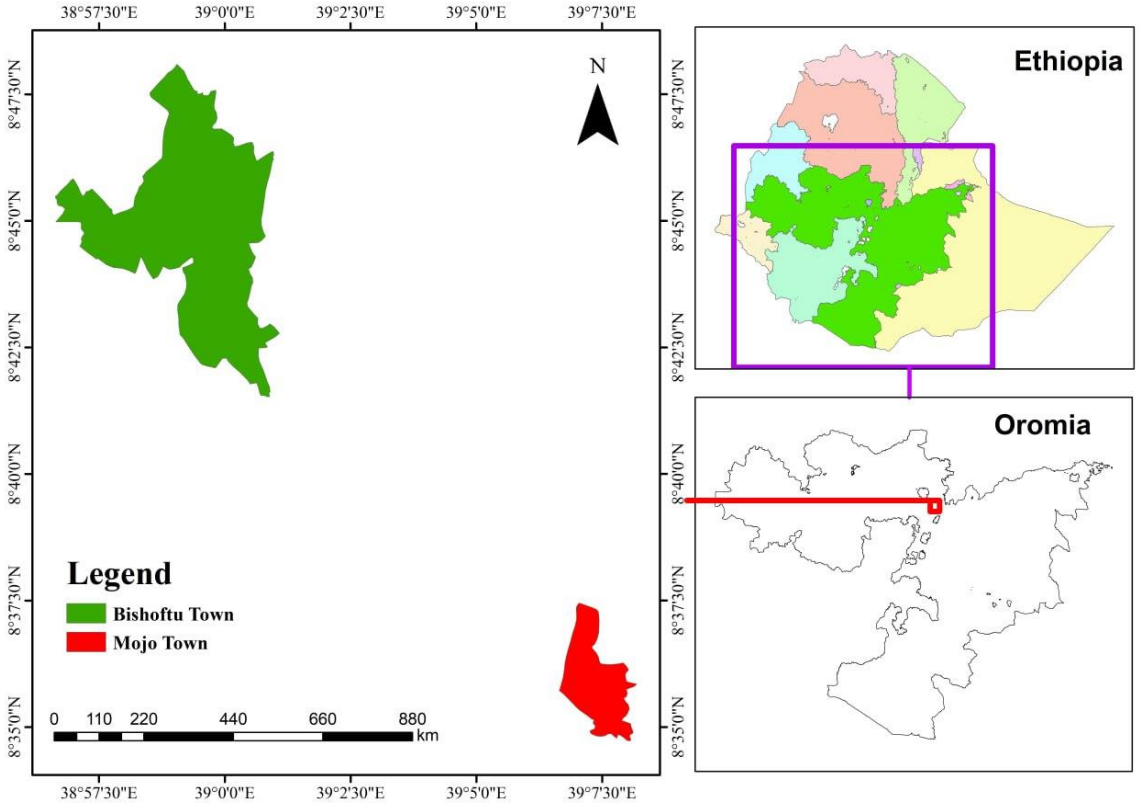

Figure 1: Map of the Study Area (developed using shapefile (Admin boundary) on ArcGIS)

3.2. The study and target populations

Commercial chicken population in Bisoftu and Mojo towns were the target population in this particular study. The study population were chickens in the sample farms. Chicken population among commercial farms in the study area were a potential sample included in the study. The study population were three breeds of exotic chickens (Bovans brown, Lohmann and Cobb 500) kept in commercial poultry farms in Bishoftu and Mojo, Central Ethiopia.

3.3. Study Animals

Chickens of different age group, sex group, and production type (layer and broiler) were the study animals. The sample chickens were of age ranges from three months to one year, both male and female sexes, broiler and layer, of the different breeds (Bovans brown, Lohmann and Cobb 500), from different farm sizes (150 to 42536 heads), and from farms with varying number of sheds or flocks (one to eight). The sample chickens were those with clinical disease, apparently healthy chicks and those died of different cases, could be infectious or non-infectious causes. They were either untreated or treated chickens with different medications such as antibiotics (Oxytetracycline, Sulfadiazine), vitamin, and anticoccidial drug, and from farms with morbidity and mortality records or not at all, vaccinated for significant poultry diseases (NCD, IB, IBD and Marek`s) or non-vaccinated ones. Generally, chicken population among commercial farms in the study area were a potential sample included in the study.

3.4. Study Design

The study design employed was a cross-sectional study. Chickens used for collection of respiratory tract swabs (oropharyngeal/tracheal swabs), cloacal swabs, and bursa and kidney tissue imprints on FAT cards were selected on the day of visit to the sample farms. A multi-stage sampling method was used in which at first the study site, Bishoftu and Mojo, were purposively selected. These two towns were selected based on the large number of intensive poultry farms located in the area. Then, sample farms were selected from both sites from which sample sheds or flocks were selected in some of the cases. Poultry farms were sampled from four directions (North, South, East and West) of Bishoftu and Mojo towns to represent all the different areas of the towns. The sample farms were selected based on convenience for the sample collection. Finally, chickens were sampled from each selected farm, or from the sample flocks. More than one shed in a farm were also sampled in some of the farms that have more than one shed. Finally, sample study units, chickens, were selected from those sample sheds in farms.

Chickens of different categories were included in the sample, where those with clinical infection of any type, if existed, were purposely included in the sample to come up with increased detection capacity and to know the circulating pathogen in the field. Swab samples were collected from these chickens sampled from different layer farms included in the study. Chickens then recently died of any problem or moribund ones, in the sample broiler farm were also sampled for the collection of tissue samples. The swab and tissue samples were then pooled in vials and FTA cards respectively as per the farm or shed from which they were collected and per their sample matrix type. The intention was to isolate, to molecularly characterize the isolated agents, and to found out the epidemiology of campylobacter, Salmonella, and viral agents of important poultry diseases (NDV, IBDV, IBV, aMPV) in sample matrix collected from farms in Bishoftu and Mojo. Further characterization of the campylobacter and the isolated viruses were conducted using sequencing and phylogenetic analysis.

3.5. Sample Size Determination

The sample size was determined based on the standard minimum sample size determination formula stated by Thrusfield (2007). The sample size for this study was calculated by considering 50% expected prevalence as there were no concise previous reports of pooled prevalence of the campylobacter, salmonella, and the viral agents to be considered for the present study. The target study population and the number of target agents were also different from the previous studies conducted in the study area and for that matter in Ethiopia. A large population that is normally distributed was assumed and a 95% confidence interval and a 5% required precision were also used for the calculation of the estimated sample size. Thus, the formula for estimation of sample size given by Thrusfield (2007),

$$n = \frac{(1.96)^2 p_{exp}(1-p_{exp})}{d^2} \ ,$$

where, n= required sample size, d= desired absolute precision, and P_{exp} = expected prevalence,

yielded the final calculated sample size of 384. To increase the representativeness of the sample, and thereby, to increase the appropriateness of the information generated, the

sample size was increased to 500. It was this final sample size that was used to incorporate chickens of different categories in the sample.

3.6. Sampling of Study Units

A purposive sampling technique was used to select study farms, sheds in farms, and study units (chickens) in the two study areas. These sampling procedures were gone on until the final sample size was fulfilled. Accordingly, a total of 500 chickens were sampled. From the total of 500 chickens, swab samples (respiratory and cloacal swabs) were collected from 400 chickens while tissue imprints were collected from the remaining 100 chickens. To generate a farm/flock level data (as poultry diseases are usually a flock problem), out of the total sample chickens, the collected samples were pooled by the farm, and flock from which it was collected and by the type of sample (matrix) collected. Based on this, a total of 54 pooled samples were collected from 16 farms located in Bishoftu (ten farms) and Mojo (six farms). There were cases in which multiple sheds were sampled when present in the farms. Accordingly, in four of the sample farms, multiple sheds were sampled (Table 11). Of the total pooled samples, 40 pools were swabs (20 respiratory swab pools and 20 cloacal swab pools), while others were tissue smears on FTA card (8 bursa and 6 kidney tissue smear pools).

The majority of the sample chickens were layers and from these birds, respiratory and cloacal swabs were collected, while bursa and kidney imprints were collected from six different sheds on a broiler farm. Chickens with clinical infection were purposely included in the sample wherever encountered. In farms with clinically infected chickens, the study units were sampled because of the presence of clinical signs such as torticollis, neck twisting, swollen eyes, eye discharge, dyspnea, salivation, diarrhea, loss of feathers, swollen vent, weakness/listlessness, depression and leg paralysis. Apparently healthy chickens were also sampled from some farms (five farms) with no clinical infection in their chickens. Sampling for the tissue smears was performed because the birds showed hyperemic bursal tissue, a swollen or atrophied bursa and urates in the kidneys at postmortem examination.

3.7. The study variables

Variables in the study were of both dependent and independent types. The dependent variable was the pathogen isolation rate while the independent variables were different, ranging from host factors to environmental factors.

3.8. Collection of Data

Data about the farms and study population were recorded along with the sample collection, in a comprehensive field data collection sheet. The data were about the number of sheds and chickens, age and genetic type, the overall mortality rate (total number of discovered dead chickens in the ongoing cycle), administered treatments, vaccination protocol, clinical signs and lesions, when present. The information about the sampled farms, flocks/sheds, and chickens, and other relevant information that was gathered along with the samples was used in the test of association between laboratory test results and those field data. The field data collected which included information about the farm of origin of the sample chickens, the management system of the chickens, flock from which they were sampled, breed, and age of the sample chickens, vaccination history, and flock health status were treated as risk factors to check against the target pathogens' isolation rates.

The mortality rates and their comparison were based on the calculated values of the rates that was obtained by dividing number of birds died in a sample farm within the time period from the introduction of a particular sample flock into the farm until the date of visit of the farm for sample collection by the total number of birds at the beginning of the introduction of the flock of the birds.

3.9. Specimen Collection and Storage

3.9.1. Collection of Swab samples and FTA card imprints

Ten respiratory (oropharyngeal and tracheal) and ten cloacal swabs were collected from each visited farm or shed on a farm. The swab samples were collected by gently introducing sterile cotton tipped applicator sticks into choanal cleft or trachea and cloaca, and gently rubbing against the mucous membrane at some angle. Before being pooled, both respiratory (oropharyngeal and tracheal) and cloacal swabs were air dried separately for ten minutes to avoid mold growth and then placed in to two different falcon vials (one for respiratory tract swab pools, and one for cloacal swab pools of a farm/shed).

Additionally, FTA card imprints of bursa of Fabricious and kidneys were collected from dead or moribund chickens, on a broiler farm in Bishoftu, where mortality was encountered at the time of visit to the farm. After dissecting the moribund chickens and locating the bursa and kidney, a small cut of the tissues with lesion were taken by thumb forceps and dusted on a sterile FTA card. The FTA card imprints were collected being pooled based on the shed or flock, and tissue type (bursa or kidney tissue).

3.9.2. Storage/ Preservation and Transportation of the Samples

The samples were stored at room temperature till packed and shipped for the molecular laboratory analyses to laboratory at the Animal Medicine, Production and Health (MAPS) Department, University of Padua (Legnaro, Italy). The samples were shipped at room temperature and they were shipped according to IATA guideline for transporting infectious agents. In the laboratory, the samples arrived were stored at −80◦C until processing, except for FTA cards, which were always kept at room temperature. Then, laboratory analyses were conducted.

3.10. Laboratory Procedures and Molecular Laboratory Tests

Each swab sample pools were resuspended in 2 mL of sterile PBS in a bigger tube and carefully vortexed before using. Then, a 200 µL aliquot of each resuspended pool was taken from the prepared solution, to be used for nucleic acid extraction. FTA card imprints were also cut, placed into 1.5 mL tubes as per their pools, resuspended in 1 mL of PBS and vortexed. The same 200 µL aliquot of each resuspended pool was taken from the prepared solution, to be used for nucleic acid extraction. For the detection of Campylobacter and Salmonella spp., DNA was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). For the viral nucleic acid extraction, a High Pure Viral Nucleic Acid Kit (Roche, Basel, Switzerland) was used.

The 200 µl aliquot for each sample pool was added (pipetted) to a separately prepared extraction buffer (lysis buffer) in an Eppendorf tube. The lysis buffer was composed of 200 µl Binding Buffer, 50 µl Proteinase K solution and 4µl poly(A). The aliquot with the lysis buffer was incubated in a thermo-block for 10 minutes to activate enzyme action. The solution was taken from the thermo-block, 100 µl binding buffer was added, and vortexed. The entire sample was transferred (pipetted) to a column tube (filter tube combined with collection tube or recovery tube) and then centrifuged at 8,000 × g for one minute (this is the centrifugation speed for the successive centrifugation steps except the last one). The flow-through in the collection tube was decanted, the filter tube and the collection tube was recombined again. Then, 500 µl inhibitor removal buffer was added, centrifuged, the flow-through was decanted, (450 µl wash buffer was added, centrifuged, decanted), the steps in the bracket was repeated once again without decanting. While the Filter Tube-Collection Tube assembly was in the centrifuge, it was spin for 10 seconds at maximum speed (approx. 13,000 × g) to remove any residual Wash Buffer (the extra centrifugation time ensures removal of residual Wash Buffer). Lastly, the Collection Tube was discarded and the Filter Tube was inserted into a nuclease- free, sterile 1.5 ml microcentrifuge tube or Eppendorf tube (another collection tube). Then the viral nucleic acids were eluted by adding 50 µl Elution Buffer to the upper reservoir of the Filter Tube, and Centrifuging at 8,000 × g for one minute. Now, the microcentrifuge tube contains the eluted, purified viral nucleic acids

to be used directly in PCR test or to be kept for later analysis. The extracted nucleic acid samples were stored either at -20 °C for brief period of time or at -80 °C until further PCR based molecular analysis.

3.11. PCR test

Based on the matrix and according to expected tropism of the different test pathogens, the extracted nucleic acids were tested with different molecular assays: those from respiratory swabs were tested for IBV, aMPV and NDV; cloacal swabs were tested for Campylobacter, Salmonella, IBV, and NDV; kidney FTA imprints were tested for IBV; while bursa FTA imprints were tested for IBDV (Table 9).

Table 7. Sample matrix and the corresponding agent it was tested for

Agent	Sample matrix			
	Cloacal swab	Respiratory swab	Kidney tissue FTA	Bursal tissue FTA
Campylobacter	√			
Salmonella	√			
NDV	√	√		
IBDV				√
IBV	√	√	√	
aMPV		√		

For *Campylobacter* spp. detection, a genus-specific PCR was performed as described by Linton *et al*. (1996), targeting the 16S rRNA gene. A genus-specific PCR performed for the detection of *Salmonella* spp., as described by Chiu *et al*. (1996), was targeting the chromosomal *invA* and plasmid *spvC* genes. The two target genes were used for rapid genus identification and evaluation of virulence, respectively. Both *Campylobacter* and *Salmonella* spp. detections were performed using the Thermo Scientific™ Phire Hot Start II DNA Polymerase kit (Invitrogen™, Waltham, MA, USA) on an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Waltham, MA, USA). In both cases, sterile water was used as a negative control while strains of *Salmonella* spp. (ATCC 700623™,

Manassas, VA, USA) and *Campylobacter jejuni* (ATCC 33560™) were used as positive controls.

The primer pairs (forward and reverse) used for amplification of the 16s rRNA for *Campylobacter* and the *invA* and plasmid *spvC* genes for *salmonella* in the PCR tests conducted, were those commercially available and routinely used in the bacteriology laboratory, University of Padua, Italy. These were also true for the viral PCR tests.

For aMPV detection and subtyping, a multiplex one-step real time RT-PCR, targeting the G gene (Cecchinato *et al*., 2013), was performed using a SuperScript™ III One-Step qRT-PCR System, with a Platinum™ Taq DNA Polymerase kit (Invitrogen™, Waltham, MA, USA) on a LightCycler® 96 Instrument (Roche, Basel, Switzerland). Using the same kit and instrument, a preliminary screening for IBV was performed, targeting the UTR region (untranslated region, a region of protein-coding genes that is transcribed into mRNA but not translated into protein) (Callison *et al*., 2006), then a one-step RT-PCR, targeting the hypervariable region of the S1 gene, was used for further characterization and sequencing of IBV-positive samples (Cavanagh *et al*., 1999). For NDV detection, a one-step RT-PCR, targeting the F gene, was used (Nanthakuma *et al*., 2000) while for IBDV, a one-step RT-PCR, targeting the VP2, was used (Jackwood *et al*., 2005). All RT-PCRs were performed using a SuperScript™ III One-Step RT-PCR System with a Platinum™ Taq DNA Polymerase kit (Invitrogen™, Waltham, MA, USA) on an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Waltham, MA, USA).

The PCR test began with a sample of the extracted nucleic acid placed in a PCR tube along with the reagents and chemicals (primers, polymerase enzyme, and nucleotide solution mix). The key components of a PCR amplification reaction are a thermostable DNA polymerase, two oligonucleotide primers, Deoxynucleotide Triphosphates (dNTPs), reaction buffer and magnesium. The tube was then placed into the PCR machine or thermal cycler. The thermal cycler takes the solution through a 3-step process: denaturation,

annealing, and extension. Finally, after taking out from the thermal cycler, amplicon presence and specificity were examined by agar gel electrophoresis in SYBR™ Safe-stained (Invitrogen™, Waltham, MA, USA) agar gel.

The electrophoresis process began by mixing molten agarose gel with intercalating dye which is SYBR® Safe stain, a cyanine dye, and pouring the gel into the casting tray with comb attached. Let the gel dry, and the comb was taken to make hole inside the dried agarose gel. The PCR product mixed with loading dye in wells of microtiter plate was added (poured) to each well formed in the agarose gel, placed in a chamber filled with conducting buffer, TBE (Tris, boric acid and EDTA) and let diffuse through the gel based on their molecular weight. The gel contains pores that allow the particles to move very slowly toward the oppositely charged side of the chamber. Finally, the gel was taken and inspected through a UV light against a known positive control and a negative control added on the two opposite ends of raw of samples poured in to the wells.

3.12. Sequencing/ phylogenetic analyses and Phylogenetic tree reconstruction

PCR products (Positive samples) were prepared and shipped to the external sequencing service of Macrogen Spain (Madrid, Spain) for purification and Sanger sequencing.
Samples positive for the various pathogens were Sanger-sequenced for species identification and strain characterization, with the same primer pairs used for PCR amplification (the RT-PCR assays) (Chiu *et al*., 1996; Cavanagh *et al*., 1999; Nanthakuma *et al*., 2000; Jackwood *et al*., 2005; Madden, 2013). Chromatogram quality was inspected with FinchTV software (Geospiza Inc., Seattle, WA, USA) and forward and reverse sequences were assembled in consensus sequences using ChromasPro 2.1.8 software (Technelysium Pty Ltd., Helensvale, QLD, Australia).

Nucleotide sequences were evaluated by BLAST search for preliminary species identification (Kumar *et al*., 2018) in Campylobacter and in order to characterize the viruses (Jackwood *et al*., 2005). BLAST search made on the Campylobacter genus-specific

PCR positive isolates was used as an initial step in campylobacter species identification using phylogenetic analysis. Sequences of Campylobacter genus positive PCR products were deposited under accession numbers OM523537-OM523550 and species identification was confirmed by phylogenetic analysis using phylogenetic tree reconstructed with the reference sequence dataset (Fig. 2).

For phylogenetic analyses, the database proposed by Valastro *et al*., (2016) was used for IBV characterization; for IBDV strain characterization, the adopted reference database and classification were those published by Islam *et al*., (2021); for NDV classification, the latest and updated classification approach by Dimitrov *et al*., (2019) was used. Sequences were deposited in Genbank and aligned to a reference dataset in MEGA X software for phylogenetic analyses. Phylogenetic trees were reconstructed using the Maximum Likelihood method, and branch support was calculated by performing 1000 bootstrap replicates (Kumar *et al*., 2018).

3.13. Data Management and Analysis

The data collected from field and the molecular laboratory tests for bacterial and the viral agents were coded and entered into Microsoft excel spreadsheets and analyzed by SPSS 26 statistical software. The major data were the different host and environmental factors, the number of samples positive for bacteria and the viral agents, and the type of agent isolated to their different level of identification. The raw data were summarized and imported to SPSS-statistics, analyzed, and interpreted.

The interpretation was usually conducted for the public health significance of the pathogens isolated from the chicken's samples. The public health significance of the pathogens was interpreted not only from results concerning isolation rates of the zoonotic pathogens but also from the field data as well as interview and personal observations conducted during sample collection stages.

The association of the different risk factors with the isolation of a specific genus/species of bacteria and virus strains was analyzed using chi-square ($\chi 2$), and logistic regression. Regression analysis for the dependent type association between Campylobacter isolation rate and that of viral agents in the swab samples was also conducted. The regression analysis conducted was a multivariable logistic regression. The interaction of multiple predictor variables was analyzed for their effect on the outcome variable, which in this case was isolation rate of the study pathogens.

A Fisher's exact probability test and Pearson chi-square test were performed (based on where they were needed) to evaluate the association between positivity and categorical test variables, whereas a Student's *t*-test and ANOVA test were used to evaluate the association between numerical test variables (age or flock size) and positivity. Taking a 5% level of significance, a p-value <0.05 was considered statistically significant. The odds ratio (OR) was also used to estimate the effect of the different risk factors on the outcome variable (isolation rate of target agents), and test dependence of Campylobacter isolation on the viral agent isolation for the same study farms.

4. RESULTS

4.1. General Overview of the Results

From a total of 500 samples in 54 pools of different matrix (respiratory tract and cloacal swabs, and bursa and kidney tissue smears on FTA card), that were collected from 16 commercial poultry farms (15 layer and one broiler farms) located in Bishoftu (10 farms) and Mojo (6 farms), 19/54 (35.2%) of the total pools were positive based on PCR test and molecular sequence analysis, for either campylobacter or the different viruses: NDV, IBDV, and IBV (Table 10).

All the respiratory tract swab pools tested for NDV, IBV, and aMPV were negative regardless of the test that has targeted for pathogens that can probably be found in respiratory system based on their tropism. The positivity for the cloacal swab pools was 14/20 (70%) and the positivity for each of the bursal tissue and kidney tissue smear pools were 3/8 (37.5%) and 2/6 (33.3%) respectively. The positive cloacal swab pools here were for either campylobacter, NDV, or IBV. Three of the 54 pools (5.6%) were found positive for more than one investigated agent (one for campylobacter and NDV, one for campylobacter and IBV, and the third for IBDV and IBV), where co-isolation was encountered. Farm level prevalence, at the same time, indicated a total positivity of 81.2% (13/16) farms for campylobacter and the viruses isolated (NDV, IBDV, IBV).

The Fisher's exact p-values for independence test between pathogen isolation and the different categorical variables in the study, results in no statistical significance association between pathogen isolation and the study variables. Odds ratio test also indicated the same odds of pathogen isolation rates between the two categories of each dichotomous independent variables (Table 10).

Table 8. Total positivity for sample pools and farms tested for bacterial and viral agents

	Variable	Total number of sample pools tested (n)	No. of positive (%)	p-value*	OR
Matrix	Bursa tissue smear	8	3 (37.5)	0.00	**
	Cloaca swab	20	14 (70)		
	Kidney tissue smear	6	2 (33.3)		
	Respiratory tract swab	20	0 (0)		
	Total	54 (pools)	19 (35.2)		
Location	Bishoftu	10	8 (80)	1.00	1.25[0.089 - 17.65]
	Mojo	6	5 (83.3)		
Genetics	Bovans brown	9	7 (77.8)	1.00	**
	Cobb 500	1	1 (100)		
	Lohmann	6	5 (83.3)		
Clinical sign	No	5	4 (80)	1.00	1.12[0.078 - 6.307]
	Yes	11	9 (81.8)		
Antimicrobial Treatment	No	3	3 (100)	1.00	**
	Yes	13	10 (76.9)		
Total		16 (farms)	13 (81.2)		

*Fisher's Exact Test ** Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Among the 16 total farms covered in the study, twelve layer farms from which cloacal swabs were collected, were positive for campylobacter, one farm for each of NDV, and IBDV (more than one shed in a farm), and two farms for IBV were also positive. The NDV positive farm and one of the two farms positive for IBV were belonged to farms already positive for Campylobacter while the second IBV positive farm was positive also for IBDV (Table 11).

From layers and broilers farms included in this study, more than one shed/flock of a farm were in case be selected in some farms, out of the different number of sheds/flocks they had (two sheds from each of three farms and three sheds from one farm). Among these, there were cases in which one shed/flock was infected with one study pathogen while the other was uninfected (this was the case in three sample farms with two sheds/flocks), while

all the three selected sheds/flocks in one sample farm were tested positive for campylobacter (Table 11).

The layer chickens' age ranged from 3 months to 1 year (mean 9.2 months), whereas broilers were 9 to 23 days old. The breed of the birds was Bovans Brown laying hens in nine farms and Lohmann laying hens in six farms and Cobb 500 for broilers. The population on the layer farms ranged from 150 to 12,000 birds (mean 2438.67) while the flock size of the total farms ranged from 150 to 42,522 birds (mean 4943.88).

Clinical sign presence was reported from four farms in Mojo and six farms in Bishoftu (10 farms in total). The administered treatments ranged from none or vitamin supplementation (seven farms) to antimicrobial drugs (oxytetracycline, sulfadiazine, norfloxacin) (eight farms), diclazuril and amprolium hydrochloride coccidiostats (one farm). Vaccination was performed in all the sample farms but the vaccination protocol varied among the farms. The vaccination protocol generally included vaccines against Newcastle disease, infectious bronchitis, infectious bursal disease, Marek's disease, fowl pox and fowl typhoid.

The mean and median overall mortality rates among birds in the sample farms were 3.25% and 0.33% (range 0–19.23%), respectively, with a higher mortality on small scale farms (rearing less than 300 birds). On some farms, no official records of mortality were kept, and farmers reported the absence of mortality that should actually have been reported as an expected number of deaths, based on the type of birds reared and management levels (Table 11).

Table 9. Information summary of data related to the sampled farms

Farm	Shed	Location	N° of Birds	Age (Month)	Breed	Clinical Signs	Mortality (%) *	Treatment	*Agent identified**
1	-	Mojo 02	150	12	Bovans brown	Twisting of neck, diarrhea	13.33	Antimicrobial drug	***C. jejuni***
2	-	Mojo 01	200	12	Lohmann	-	0	Not administered	***C. jejuni, NDV***
3	1	Mojo 02	260	12	Bovans brown	Diarrhea, depression	19.23	Ab, Vit.	***C. avium***
	2								-
4	-	Mojo 01	350	12	Lohmann	Depression, weakness	0	Not administered	***C. avium***
5	-	Mojo 02	450	12	Bovans brown	Diarrhea, swollen eyes	0.44	Ab, Vit.	-
6	1	Mojo 01	500	12	Lohmann	-	0	Not administered	-
	2			7					***C. jejuni***
7	-	Bishoftu 05	570	10	Lohmann	Diarrhea, twisting of the neck	5.61	Ab	***C. jejuni***
8	-	Bishoftu 05	800	5	Bovans brown	Dyspnea	0.12	Vitamin	-
9	-	Bishoftu 09	1800	3	Lohmann	-	0.83	Vit.	-
10	-	Bishoftu 01	2500	12	Lohmann	-	4	Ab,Vit.	***C. jejuni***
11	-	Bishoftu 05	3000	5	Bovans brown	-	0.13	Vit.	***C. jejuni***
12	-	Bishoftu 09	3000	3	Bovans brown	Diarrhea, depression	0.33	Anticoccidial, Vit.	***C. jejuni, IBV***
13	1	Bishoftu 05	5000	12	Bovans brown	Diarrhea, neck twisting, leg paralysis	1.00	Vitamin supply	***C. jejuni***
	2								-
14	-	Bishoftu 01	6000	9	Bovans brown	Torticollis, swollen eyes, diarrhea,	3.33	Vitamin supply	***C.avium***
15	1	Bishoftu 05	12,000	12	Bovans brown	Discharges from eye, dyspnea, salivation	0.33	Ab, Vit.	***C. jejuni***
	2								***C. jejuni***
	3								***C. helveticus***
16	1	Bishoftu 02	42,522	1	Cobb 500	Post mortum lesions	0.07	Antimicrobial drug	***IBDV***
	2								***IBDV***
	5								
	6								***IBDV, IBV****
	9								
	10								***IBV***

* Overall mortality rate for the ongoing production cycle.
Ab - Antimicrobial drug Vit. - Vitamin supply
* two different pools (a bursa and a kidney tissue smear pools) from a single shed
* Agent identified based on PCR detection of pathogen and sequencing tests

4.2. Results about Campylobacter and Salmonella

Twenty cloacal swab pools taken from sample chickens from fifteen layer farms has been tested for Salmonella. All these samples tested for *Salmonella* spp. were negative. Though extracts from each cloacal swab pools were tested for campylobacter, IBV, NDV, and salmonella separately using different PCR tests, exceptionally no one pool has been tested positive for salmonella, even though variable number of swab pools were tested positive for the remaining tested pathogens.

The farm-level positivity for campylobacter isolation was 80.0% (12 out of 15 farms tested for campylobacter) (Table 12). There were only three farms in general that were tested negative to campylobacter. The odds for campylobacter isolation rates didn't show variations between the categories of the different test variables included in the study. The Fisher's exact test also shows non-significant results (p-values = 1) for independence test between campylobacter isolation and the different binary categorical variables in the study. Odds ratio test also indicated that there is no difference in odds of campylobacter isolation between the two categories of each dichotomous factor variables.

Table 10. Number of Campylobacter test positive farms for different test variables based on PCR test

Variable	Total farms (n)	No of positive (%)	p-value	odds ratio [CI]
Location				
Bishoftu	9	7 (77.8)	1.000	1.429 [0.100 – 20.437]
Mojo	6	5 (83.3)		
Breed				
Bovans	9	7 (77.8)	1.000	1.429 [0.100 – 20.437]
Lohmann	6	5 (83.3)		
Treatment status				
Do not use AM	3	3 (100.0)	1.000	**
Use AM	12	9 (75.0)		
Clinical sign				
Absent	5	4 (80.0)	1.000	1.000 [0.068 - 14.640]
Present	10	8 (80.0)		
Total	15	12 (80.0)		

** not calculated for

The age of birds at time of sampling, number of birds per farm, and mortality rate (calculated for each sample farm from number of dead birds as compared to total flock size), were summarized to compare their mean values between test positive and negative farms from which the samples were collected (Table 13). Accordingly, the mean age of the birds of the sample farms was 10 months for the positive farms and 7 months for the negative farms. The mean flock size of the positive farms was 2794 while that of the negative farms was 1017. The comparison between positive and negative farms (t-test) in terms of age mean and flock size mean yielded a not statistically significant difference ($p = 0.18$; $p = 0.41$). The mean mortality rate in the farms from which test positive cloacal samples were collected was 3.93% while that of the negatives was 0.47%. The mean and median overall mortality rates in the farms enrolled in the study were 3.48% and 0.39% (range 0.00–19.23%), respectively, with higher rates in smaller farms (farms n. 1, 2, 3; <300 birds). But there was no statistically significant association between mortality rate and campylobacter positivity (p =0.36).

However, the number of deaths reported by each farm owners and attendants were the recalls of the respondents as there were no concrete compiled farm records to refer as a secondary data source. Due to this, some respondents have reported no death in a farm, and thereby the mortality rates were taken to be zero. The absence of mortality reported in some farms should be interpreted in light of the lack of rigorous recording methods. Mortality rates should be rather intended as an average rate of mortality based on the hybrid type of the birds, production and management in the farms in the absence of acute causes.

Table 13. Average Number of different test variables for Campylobacter test positive farms

Variable	Mean of positive farms	Mean difference between positive and negative	p-value
Number of sheds	2.33	1.33	0.305
Flock size	2794.17	1777.5	0.412
Age of chickens	9.83	3.167	0.181
Mortality rates	3.93	3.466	0.362

Campylobacter test for pool of samples showed that 14 out of 20 pooled samples (70.0%) tested for *Campylobacter* spp. were positive. This became 9 positive cloacal swab pools from a total of 12 cloacal sample pools from Bishoftu (9/12, 75%) and 5 positive pools of cloacal sample out of 8 total cloacal swab pools from Mojo (5/8, 62.5%) (Table 14). From six pools of samples that were collected from farms with apparently healthy birds (with no clinical sign), 4 of them were tested positive whereas, from 14 pooled samples collected from farms having clinically infected birds with any poultry diseases, 10 pools were tested positive for campylobacter. On the other hand, one out of five farms not reporting clinical signs was negative for *Campylobacter* spp., whereas two out of ten farms with clinical signs were negative (Table 14). It is seven out of eight farms reporting enteric signs that were positive. That is, the number of Campylobacter test positive cloacal swab pools were higher for the sample farms that have birds with clinical disease than their corresponding sample farms without clinical sign. But there was no statistically significant association between clinical signs and positivity (p = 1.00). The number of Campylobacter test positive

cloacal swab pools were also higher than their corresponding test negative pools for both of the farms that have used antimicrobial drugs and those that didn't. However, there was no statistically significant association between antimicrobial drug use and positivity for *Campylobacter* spp. (p = 1.00). The p-values and odds ratios have indicated that there is no statistically significant dependence of isolation of tested pathogens and different variables included in the study.

Table 14. Campylobacter test positivity for pools of samples among different test variables based on PCR test

Variable	Total number of farms tested (n)	No of positive (%)	p-value	odds ratio [CI]
Location				
Bishoftu	12	9 (75.0)	0.642	0.556 [0.080 – 3.858]
Mojo	8	5 (62.5)		
Breed				
Bovans	13	9 (69.2)	1.000	1.111 [0.148 – 8.367]
Lohmann	7	5 (71.4)		
Treatment status				
Do not use AM	4	3 (75.0)	1.000	0.733 [0.060 - 8.915]
Use AM	16	11 (68.8)		
Clinical sign				
Absent	6	4 (66.7)	1.000	1.250 [0.160 - 9.765]
Present	14	10 (71.4)		
Total	20	14 (70)		

The t-test computed for pools of samples to compare distribution of campylobacter positivity based on means of both age and mortality, each compared between their respective positive and negative pools, resulted in no statistically significant differences (p=0.63; p=0.95). The mean age of the birds that belonged to farms from which the positive sample pools were collected were not significantly different from the mean age of the sample birds taken from Campylobacter test negative farms. Besides, the average mortality rate in the Campylobacter test positive farms from which the sample pools were collected was not significantly different from the average mortality rate in the test negative farms. In

the same way, the mean flock size compared between test positive and negative pools of cloacal samples didn't show a significant association (p = 0.08) between flock size and Campylobacter positivity (Table 15).

Table 15: Pools of sample`s distribution of Campylobacter test positivity

Variable	Mean of positive pools	Mean difference between positive and negative pools	p-value
Number of sheds	3.14	1.143	0.368
Flock size	4109.29	2640.952	0.085
Age of chickens	10.14	0.810	0.631
Mortality rates	3.42	-0.182	0.954

4.3. Campylobacter species identified and results of the Sequencing and Phylogenetic Analysis

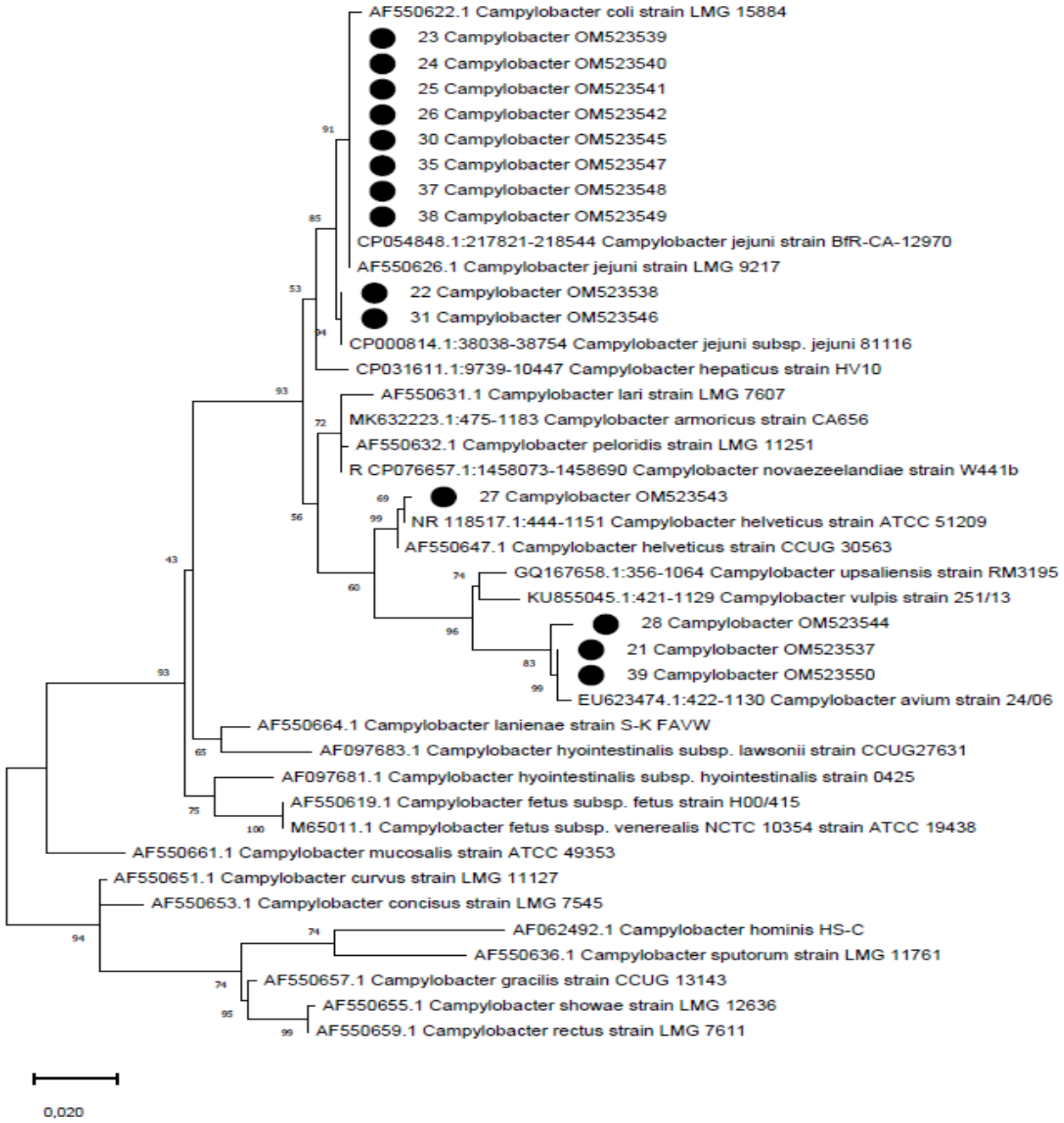

Figure 2: Phylogenetic tree reconstructed based on 16S ribosomal RNA gene partial sequences for Campylobacter

The Campylobacter genus positive PCR products in this study were deposited under accession numbers OM523537-OM523550. The strains identified based on the sequencing and phylogenetic analysis were those marked with a black circle in the above figure (Fig. 2). The phylogenetic tree was reconstructed using the maximum likelihood method, with the Kimura 2-parameter and general time reversible model with discrete gamma distribution. Branch support is shown next to the branches.

Based on the phylogenetic analysis, 14 isolates that were belonged to three species of Campylobacter namely *C. jejuni*, *C. avium* and *C. helveticus* were identified. The *C. jejuni* has 99.58–100% identity with reference strain BfR-CA-12970 (Acc. Num. CP054848.1), the *C. avium* has 99.03–99.72% identity with reference strain 24/06 (Acc. Num. EU623474.1), and the *C. helveticus* has 99.86% identity with reference strain ATCC 51209 (Acc. Num. NR_118517.1). Within the *C. jejuni* isolates (10 isolates), there were variabilities in which eight of them exactly align with reference strain BfR-CA-12970 while the other two isolates rather align with a different *C. jejuni* strain (LMG 9217), showing deviation from the reference strain and resulting in an identity less than 100% with respect to the reference strain taken (Figure 2).

Of the twenty cloacal sample pools tested for campylobacter, 14 of them were found positive. From these totals of fourteen (14) Campylobacter test positive cloacal swab pools, ten (10) sample pools (71.43%) that belonged to nine (9) different farms were positive for *C. jejuni* (Table 15). Two *C. jejuni* positive pools were from two different sheds/flocks of a single farm, and that is why a farm level *C. jejuni* positivity (9/12, 75%) is different from the positivity (10/14, 71.43%) for the pools of samples. Three sample pools (3/14, 21.43%) that belonged to three different farms were positive for *C. avium* (with 3/12, 25% farm-level *C. avium* test positivity), and one sample pool (1/14, 7.14%) was positive for *C. helveticus* (with 1/12, 8.33% farm-level test positivity for *C. helveticus*). *Campylobacter helveticus* positive sample pool was obtained from a farm that was positive also for *C. jejuni* but identified from a different sample pool from another shed in the farm. No cloacal sample pool became positive for more than one campylobacter species but two pools from different sheds in one farm did.

In this study, there were nine *C. jejuni* positive farms from the total of twelve Campylobacter test positive farms (9/12, 75% farm-level positivity for *C. jejuni*). This mean, *Campylobacter jejuni* was isolated from more than half of the sample farms (9/15, 60%), and together with *C. avium* they were accounted for (12/15, 80%) farm positivity. This is the same as the total farm positivity for Campylobacter genus (Table 12). Here, one farm was double counted for *C. jejuni* and *C. helveticus* and one farm in Bishoftu had its two sheds/flocks positive for *C. jejuni*, and that is why the number of positive farms became nine (one lower than the number of positive pools which was ten in number) (Table 16).

Table 16: Sample pool and Farm level distribution of the campylobacter spp. isolated

		Species				
	Pool	*C. avium*	*C. helveticus*	*C. jejuni*	Negative	Total
Total	Count (%)	3 (15)	1 (5)	10 (50)	6 (30)	20 (pool)
	Farm	*C. avium*	*C. jejuni* and *C. helveticus*	*C. jejuni*	Negative	Total
Total	Count (%)	3 (20.0)	1 (6.7)	8 (53.3)	3 (20.0)	15 (farms)

Half of the negative sample pools (3/6) were collected from farms where more than one shed was sampled (farms no. 3, 6, 13, 15) (Table 11). The *C. helveticus* was detected in one shed of farm 15, where multiple sheds were sampled (three sheds), and the two other sheds were positive for *C. jejuni*. In two farms (farms 6 and 13), a shed was positive for *C. jejuni*, and the other was negative, and in another farm (farm 3), *Campylobacter* (*C. avium*) was detected only in one of the two sheds. This means, there are variabilities in isolation rates of campylobacter genera and the different species between and among sheds in single study farm.

Six *C. jejuni* positive farms (6/15, 40%) were in Bishoftu and three farms (3/15, 20%) were in Mojo. Five *C. jejuni* isolates were from Bovans brown chickens (four isolates from farms in Bishoftu and one from Mojo), while four isolates of *C. jejuni* were from Lohmann chickens (three isolates from Bishoftu and one from Mojo). The three *C. avium* isolates were isolated from two farms in Mojo (one Bovans brown and one Lohmann chickens

rearing farms) and one farm in Bishoftu (with Bovans brown layer chickens). The only *C. helveticus* isolate was detected from a sample pool collected from one shed/flock of Bovans brown layers farm in Bishoftu that had eight different sheds/flocks at the time of the study. This species was co-isolated with *C. jejuni* that was isolated from two different sheds/flocks of the same farm. This farm was unique among other sample farms with its number of sheds/flocks, and with the then suspected upcoming storm of disease outbreak.

Seven *C. jejuni* test positive swab pools were the one collected from poultry farms in Bishoftu while three of them were obtained from farms in Mojo. Six of the *C. jejuni* test positive swab pools were the one collected from poultry farms with Bovans brown layers farm while four of them were from Lohmann layers farms. The *C. avium* test positive cloacal swab pools were collected from farms in Bishoftu and Mojo as well as from both Bovans brown and Lohmann layers farms. Two out of the three *C. avium* test positive cloacal swab pools were belonged to pools collected from poultry farms in Bishoftu, and Lohmann layers farms in both areas. The *C. jejuni* test positive cloacal sample pools were mainly obtained from farms whose poultry were with clinical diseases. Six *C. jejuni* test positive pools were from farms with clinically infected birds, among ten sample pools positive for *C. jejuni* altogether. An equal number of cloacal swab pools became test positive for *C. jejuni* out of the sample pools collected from both groups of farms in terms of their antimicrobial use (Table 11).

Based on ANOVA test to compare means of different numerical variables among three groups of the dependent variable, campylobacter isolation rates (*C. jejuni*, *C. avium* and test negatives), both for the sample farms and pools of samples yields the following: The mean flock size for *C. jejuni* positive farms was 2991 while that of *C. avium* positive farms was 2203. The mean mortality rate for *C. jejuni* test positive farms was 2.74% while that of the *C. avium* test positive farms was 7.5%, but the average age for the two groups was similar (9.4 and 11 months). For the pool of samples as well, variabilities were observed among the categories but none of them have shown a statistically significant difference just like the farm data (Table 17).

Table 17. Mean values for the test variables regarding *C. jejuni* and *C. avium* test positivity

		No. of Sheds	No. of Birds	Age	Mortality
		Mean	Mean	Mean	Mean
Spp. (farm)	*C. jejuni*	2.56	2991.11	9.44	2.74
	C. avium	1.67	2203.33	11.00	7.50
	negative	1.00	1016.67	6.67	0.4667
p-value		0.483	0.681	0.342	0.309
Spp. (pool)	*C. jejuni*	3.10	3892.00	9.70	2.50
	C. avium	1.67	2203.33	11.00	7.50
	negative	2.86	2972.86	9.71	3.1329
p-value		**0.711**	**0.813**	**0.840**	**0.492**

4.4. Results on Viral Agents Investigation and Characterization

The investigation made on important viral pathogens in poultry (Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), and avian metapneumovirus (aMPV)) using biomolecular assays and sequencing, results in low isolation rates. Three bursal tissue smear pools that belonged to three different sheds of a single broiler farm, were tested positive for IBDV and two kidney tissue smear pools were tested positive for IBV that together amounted for a total positivity of 5 (35.7%) out of the 14 tissue smear pools tested for the two viruses based on their tropism (bursa tissue for IBDV and kidney tissue for IBV). In addition to kidney tissue smear pools, IBV was tested for respiratory tract and cloacal swab pools, but only one cloacal swab pool was found positive for IBV.

Table 18: Number of positive sample pools and distribution of positivity for the viruses

Variable	Total number of pools tested (n)	No of positive (%)	p-value	odds ratio [CI]
Matrix				
Bursa tissue	8	3 (37.5)	1.000	0.833 [0.090 – 7.675]
Kidney tissue	6	2 (33.3)		
Total	14	5 (35.7)		
Respiratory tract swab	20	0		
Cloacal swab	20	2 (0.1)		

		Pathogen type				
		IBDV	IBV	NDV	aMPV	Total
Tissue	Bursa Count (%)	3 (37.5)	-	-	-	8
	Kidney Count (%)	-	2 (33.3)	-	-	6
Swab	Cloaca Count (%)	-	1 (5.0)	1 (5.0)	-	20
	Respiratory tract	-	0	0	0	20

For the aMPV test, all the twenty pools of respiratory tract swab samples were test negative, may be due to one or more of the reasons mentioned above. Again, for the NDV tests, all the respiratory tract swab pools were negative, while only one cloacal swab pool from a Lohmann layers farm of 1-year-old birds, located in Mojo town was tested positive (Table 11). The NDV positivity was therefore, 1/15 layers farms, 6.7% or 1/40, 2.5 % sample pools tested for NDV (20 respiratory tract and 20 cloacal swab pools).

For the IBV test, a total of 2 out of 16 farms (12.5%) (three sample pools: one cloacal swab pool from layers farm and two FTA card kidney imprints from the broiler farm) were positive from a total of 46 pools tested for IBV (20 respiratory tract and 20 cloacal swab pools and 6 kidney tissue smear pools). Regarding IBDV test results, 3 out of 8 bursa imprint pools from the broiler farm (1/16 farms, 6.25%), were positive (Table 18).

The isolated NDV was a vaccine strain close to the Lasota strain (showing a 99.8% identity with strain ID AF077761) belonging to genotype II, based on the updated classification (Dimitrov *et al*., 2019) (GenBank accession no. AF077761, class II, genotype II) (Figure 3). This vaccine was used in the vaccine protocol implemented on the positive farm, as reported by the farmer.

The NDV strain characterized in the study was the one marked with a red circle (Fig 3). The phylogenetic tree was reconstructed using the Maximum Likelihood method and Kimura 2-parameter model with discrete Gamma distribution. Branch support is shown next to the branches.

Figure 3: Phylogenetic tree reconstructed for the characterized NDV strain with reference to the database proposed by Dimitrov *et al*., (2019)

The IBDV test resulted in highly similar sequences (99.8–100% identity) to the Winterfield-2512 vaccine strain (reference strain MH329181.1), belonging to the classical/virulent genogroup A1a (Islam *et al*., 2021) (Figure 4). According to the declared vaccination strategy of the farm, this strain was the one used for the bird immunizations.

The Ethiopian strains isolated in this study were those marked with a red circle in the figure bellow (Fig. 4). The phylogenetic tree reconstructed for IBDV strain characterization was reconstructed according to the database proposed by Islam *et al*., (2021). It was reconstructed using the Maximum Likelihood method and Kimura 2-parameter model with a discrete Gamma distribution. Branch support is shown next to the branches. Two of the three strains of IBDV isolated in the study, have 100% similarity with the reference strain while one of them has a lesser similarity with the reference strain used.

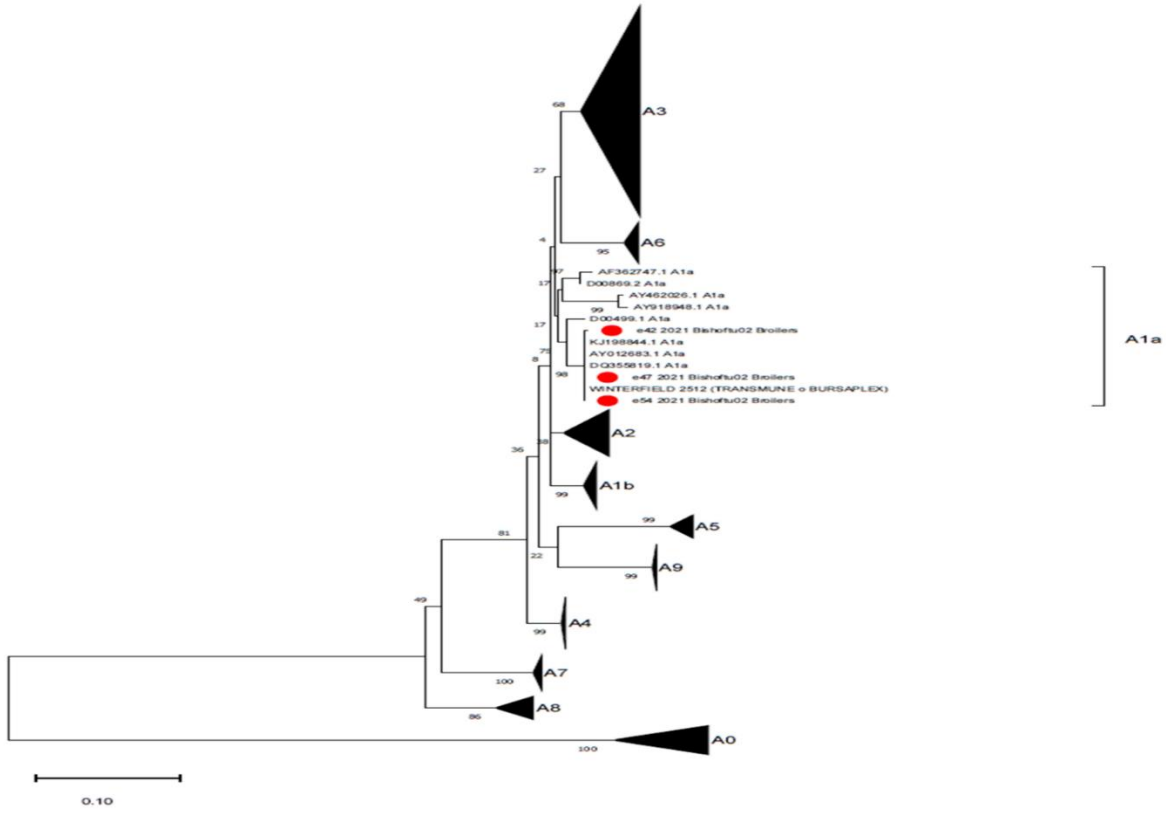

Figure 4: Phylogenetic tree reconstructed for IBDV strain characterization with the database proposed by Islam *et al*., (2021)

For IBV, based on real-time RT-PCR screening test at high Ct (>38), two IBV test positive kidney tissue smear pools were detected. However, due to sensitivity limits, only one sample was successfully sequenced for IBV, resulting in a 4/91-like (793B) strain (GI-13) (Valastro *et al*., 2016) (shows a 99.4% identity with the reference strain MT701511.1) (Figure 5). This sequenced IBV positive sample was the one isolated from cloacal swab pool collected from a layer farm located in Bishoftu town hosting 3-month old chickens with gastroenteric clinical signs and depression. It was reported that these birds were vaccinated with 1/96-based (GI-13) and mass-based (GI-1) vaccines (Table 11).

The Ethiopian IBV strain isolated was one marked with a red circle (Fig. 5). In the bellow phylogenetic tree, sequences belonging to the different lineages have been collapsed and single branches represent unique variants. The phylogenetic tree was reconstructed using the Maximum Likelihood method and General Time Reversible model with discrete Gamma distribution. Branch support is shown next to the branches.

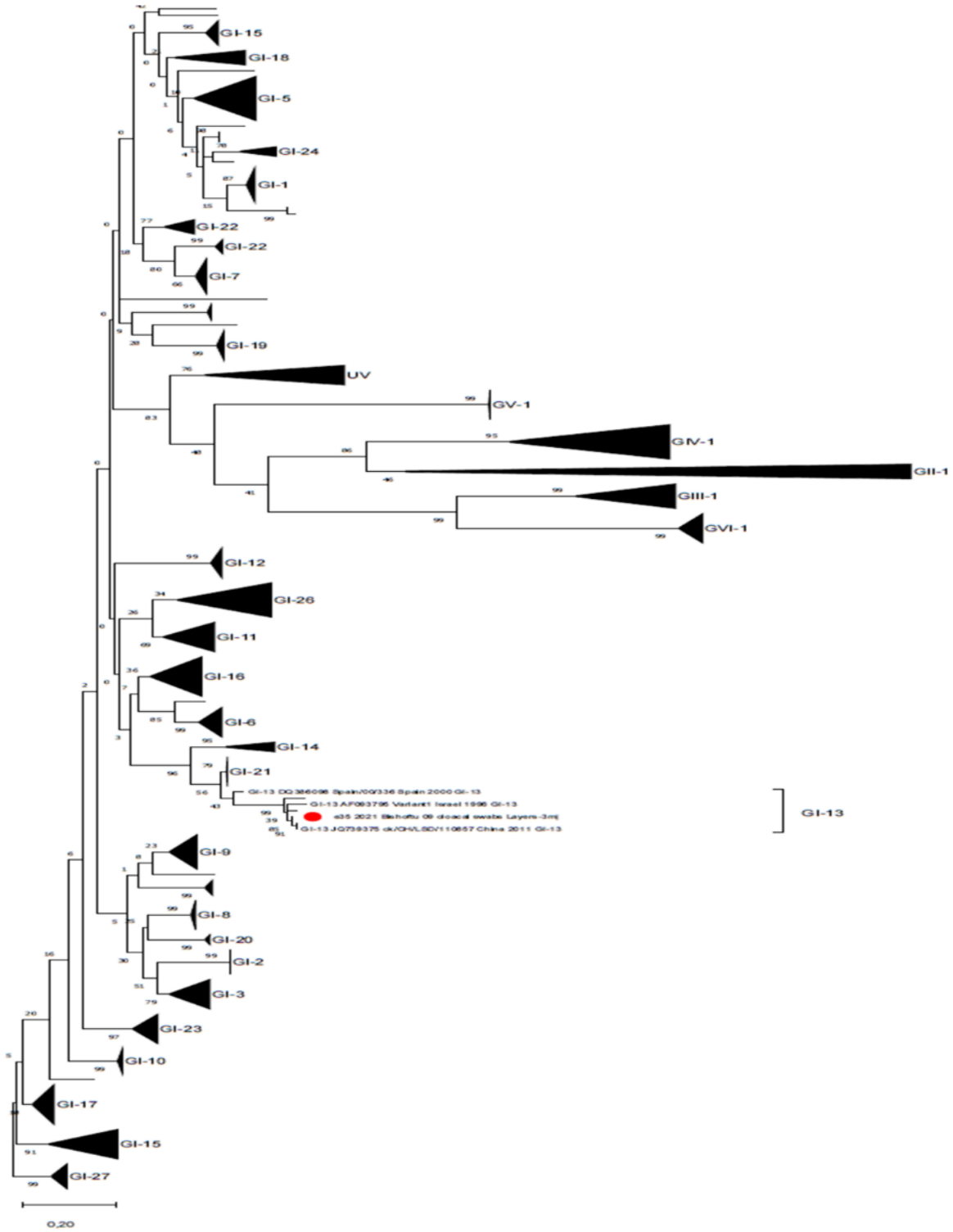

Figure 5: Phylogenetic tree reconstructed for IBV strain characterization with the database proposed by Valastro *et al*., (2016)

Comparison between the two viruses isolated from cloacal sample pools (IBV and NDV) against the different test variables showed that IBV was isolated from Bovans brown while NDV was isolated from Lohmann layer farms. The NDV was isolated from a farm with apparently healthy chickens flock while IBV was isolated from cloacal swab pool collected from a farm with clinically infected chickens. Both viruses were isolated from farms that had used antimicrobial drugs while NDV was from a Newcastle disease vaccinated farm. The farm from which IBV was isolated had used vaccines against infectious bronchitis, Marek's disease and fowl pox in addition to the Newcastle disease vaccine. Both the viruses isolated from cloacal swab pools were co-isolated with *C. jejuni* (Table 11).

4.5. Epidemiological Analysis of the study variables

The total farm-level positivity for the test variables (pathogen isolation) in the present study was 13/16 (81.25%). This was regardless of the pathogen type tested for and the different variables included in the study. With respect to the study sites, 8/10 (80%) farms in Bishoftu and 5/6 (83.3%) farms in Mojo were tested positive for either bacteria (Campylobacter) or virus (NDV, IBDV, IBV). Eighty percent (80%) of the farms tested for Campylobacter were positive (12/15, 80%), while 70% of the pooled cloacal samples were test positive (14/20, 70%). Of the Campylobacter test positive farms, 7/12 (58.3%) were belonged to Bishoftu and 5/12 (41.7%) farms belonged to Mojo. Seven out of nine farms in Bishoftu that were tested for Campylobacter were positive (7/9, 77.8%) while five out of six farms sampled from Mojo were tested positive for Campylobacter (5/6, 83.3%). One IBV positive farm from Bishoftu and one NDV positive farm from Mojo were also included among the Campylobacter test positive farms (Table 11).

For the viral pathogens, a total of three farms (2 layers farms and the broiler farm) were tested positive. Among these, the broiler farm takes the largest share with 3 IBDV and 2 IBV isolates. The two layers farms accounted each for one isolate (1 IBV and 1 NDV). The viruses isolated from layers farms were obtained from cloacal swab pool. On the other hand, 14 pooled tissue smear samples tested for virus isolation, yielded in 3 positive pools

for IBDV and 2 positive pools for IBV. All the three IBDV positive pools were belonged to three different sample sheds while the two IBV positive sample pools were collected from two different sheds of the broiler farm but one of these sheds was among the three sheds that were positive for IBDV.

Table 19. Isolation rate of Campylobacter bacteria and viruses (IBDV, IBV, NDV)

		Total isolation by pathogen			Pathogen type			
					Bacteria	Virus	Bacteria and Virus	p-value
		Total (n)	Positive no. (%)	p-value	positive	positive	positive	
Matrix	Bursa	8	3 (37.5)	0.000	-	3 (37.5)	-	0.000
	cloacal	20	14 (70.0)		12 (60.0)	-	2 (10.0)	
	kidney	6	2 (33.3)		-	2 (33.3)	-	
	tracheal	20	0(0)		-	0	-	
Location	Bishoftu	38	14 (36.8)	0.764*	8 (21.1)	5 (13.2)	1 (2.6)	0.451
	Mojo	16	5 (31.2)		4 (25.0)	-	1 (6.2)	
Genetics	Bovans	27	9 (33.3)	0.950	8 (29.6)	-	1 (3.7)	0.002
	Cobb	13	5 (38.5)		-	5 (38.5)	-	
	Lohmann	14	5 (35.7)		4 (28.6)	-	1 (7.1)	
Sign	No	12	4 (33.3)	1.000*	3 (25.0)	-	1 (8.3)	0.497
	Yes	42	15 (35.7)		9 (21.4)	5 (11.9)	1(2.4)	
treatment	no	8	3 (37.5)	1.000*	2 (25.0)	-	1 (12.5)	0.412
	Yes	46	16 (34.8)		10 (21.7)	5 (10.9)	1 (2.2)	

The IBV and NDV test positive farms were layers farms sampled from Bishoftu with Bovans brown chickens and from Mojo with Lohmann breeds of chickens respectively. The prevalence of Campylobacter being compared between and among different categories of the study variables, showed that the prevalence was not significantly different from each other with in their respective categories ($p > 0.05$) for all the different categories of study variables. That is, the odds of campylobacter isolation in one category of the test variables was not significantly different from the other categories of its group. In all the cases, the prevalence of Campylobacter was higher or equal to 50%. The total farm and pool prevalence were also compared to each other and resulted in a non-significant difference ($p > 0.05$) (Table 20).

Table 20. Proportion of farm and pool positivity for Campylobacter against study variables

Study Variables		**Number of farms/pools examined**	Number of farms/pool positive	Prevalence (farm/pool) %	***p*-value (farm/pool)**
Site/Location	Bishoftu	9(12)	7(9)	77.8(75)	**1.000**
	Mojo	6(8)	5(5)	83.3(62.5)	**(0.642)**
Breed type of birds	Bovans	9(13)	7(9)	77.8(69.2)	**1.000**
	Lohmann	6(7)	5(5)	83.3(71.4)	**(1.000)**
Age of birds at sampling	Chick (0-4 mth]	2(2)	1(1)	50(50)	**0.216**
	Pullet (4-6 mth]	2(2)	1(1)	50(50)	**(0.621)**
	Hen (>6 mth)	11(16)	10(12)	90.9(75)	
Number of birds per farm (flock size)	(50-500] (small scale)	6(8)	5(5)	83.3(62.5)	**0.812**
	(500-10,000) (medium scale)	8(9)	6(6)	75(66.7)	**(0.461)**
	>= 10,000 (large scale)	1(3)	1(3)	100(100)	
Mortality	Yes	12(16)	9(11)	75(68.8)	**1.000**
	No	3(4)	3(3)	100(75)	**(1.000)**
Clinical sign	Yes	10(14)	8(10)	80(71.4)	**1.000**
	No	5(6)	4(4)	80(66.7)	**(1.000)**
Antimicrobial	Yes	6(9)	5(7)	83.3(77.8)	**1.000**
	No	9(11)	7(7)	77.8(63.6)	**(1.000)**
Total		15(20)	12(14)	80(70)	**1.000***

***** compared between farm and pool prevalence

Campylobacter's species distribution with respect to different test variables' categories was also analyzed. Based on this, 2/3 (66.7%) *C. avium* positive farms were in Mojo, one farm was with Bovans brown and the other one was with Lohmann laying hens. Ten, 10/14 (71.4%) of the Campylobacter positive isolates belonged to *C. jejuni* species where 7/10 (70%) were from Bishoftu and the remaining 3/10 (30%) were from Mojo. The rate of isolation of the three Campylobacter species isolates were 50% for *C. jejuni*, 15% for *C. avium*, and 5% for *C. helveticus*. The test for significant difference among the isolation rate of the three species, resulted in a non-significant p-value ($p > 0.05$), indicating a statistically non-significant difference in isolation rate among the Campylobacter isolates of the present study (Table 20).

Table 21: Campylobacter spp. isolation between and among categories of factor variables

		Species				
		C. avium	*C. helveticus*	*C. jejuni*	Test negative	p-value
Location	Bishoftu	1 (8.3)	1 (8.3)	6 (50)	3 (25)	
	Mojo	2 (25.0)	0 (0)	3 (37.5)	3 (37.5)	0.620
Total (pool)		3 (15.0)	1 (5.0)	9 (45.0)	6 (30.0)	
		Species				
		C. avium	*C. helveticus* & *C. jejuni*	*C. jejuni*	Test negative	p-value
Location	Bishoftu	1 (11.1)	1 (11.1)	5 (55.6)	2 (22.2)	
	Mojo	2 (33.3)	0 (0)	3 (50.0)	1 (16.7)	0.652
Total (farm)		3 (20.0)	1 (6.7)	8 (53.3)	3 (20.0)	
		Species				
		C. avium	C. helveticus	C. jejuni	Test negative	p-value
genetics	Bovans	2 (15.4)	1 (7.7)	6 (46.2)	4 (30.8)	
	Lohmann	1 (14.3)	0 (0)	3 (42.9)	2 (28.6)	0.659
Total (pool)		3 (15.0)	1 (5.0)	9 (45.0)	6 (30.0)	
		Species				
		C. avium	*C. jejuni* & *C. helveticus*	*C. jejuni*	Test negative	p-value
genetics	Bovans	2 (22.2)	1 (11.1)	4 944.4)	2 (22.2)	0.774
	Lohmann	1 (16.7)	0 (0)	4 (66.7)	1 (16.7)	
Total (farm)		3 (20.0)	1 (6.7)	8 (53.3)	3 (20.0)	

Different types of sample pools were tested for presence of different viral agents, as per their tropism. All the 54 sample pools (20 cloacal and 20 respiratory swab pools, 8 bursal and 6 kidney tissue imprint pools) were tested for viral agents, in general. Forty (40) swab pools were tested for NDV, 8 total pools (8 bursal tissue FTA card imprint pools) were tested for IBDV, and 46 total pools (40 cloacal and respiratory swab and 6 kidney tissue imprint pools) were tested for IBV (Table 21). Of these, one cloacal swab pool was tested positive for NDV, three bursal tissue imprint pools were tested positive for IBDV, and three pools (one cloacal and two kidney tissue imprint pools) were tested positive for IBV. The analysis of prevalence of the three viral agents isolated in the present study has showed a significantly different isolation rate for the viruses ($p < 0.05$).

Table 22 Isolation rate of Campylobacter, NDV, IBDV and IBV

		Pathogen type						
		IBDV	IBV	IBV-campylo	NDV-campylo	Campylobacter	Total	p-value
matrix	Bursa	3 (37.5)	0 (0)	0 (0)	0 (0)	0 (0)	8	**0.000**
	cloacal	0 (0)	0 (0)	1 (5.0)	1 (5.0)	12 (60.0)	20	
	Kidney	0 (0)	2 (33.3)	0 (0)	0 (0)	0 (0)	6	
	pharyngeal	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	20	
Total (pool)		3 (5.6)	2 (3.7)	1 (1.9)	1 (1.9)	12 (22.2)	54	
Location (pool)	Bishoftu	3 (7.9)	2 (5.3)	0 (0)	1 (2.6)	8 (21.1)	38	0.412
	Mojo	0 (0)	0 (0)	1 (6.2)	0 (0)	4 (25.0)	16	
		Group of Pathogen						
		Campylobacter		IBV-Campylo	IBV-IBDV	NDV-Campylobacter		p-value
location	Bishoftu	6 (60.0)		1 (10.0)	1 (10.0)	0 (0)		**0.028**
	Mojo	4 (66.7)		0 (0)	0 (0)	1 (16.7)		
Total (farm)		10 (58.8)		1 (5.9)	1 (5.9)	1 (5.9)		
		Pathogen type						
		IBDV	IBV	IBV-campylo	NDV-campylo	campylobacter	Total	p-value
Genetics (pool)	Bovans	0 (0)	0 (0)	0 (0)	1 (3.7)	8 (29.6)	27	**0.009**
	Cobb	3 (23.1)	2 (15.4)	0 (0)	0 (0)	0 (0)	13	
	Lohmann	0 (0)	0 (0)	1 (7.1)	0 (0)	4 (28.6)	14	
		Group of Pathogen						
		Campylobacter		IBVCampylo	IBVIBDV	NDVCampylo		p-value
Genetics (Farm)	Bovans	6 (66.7)		1 (11.1)	0 (0)	0 (0)		**0.001**
	Cobb	0 (0)		0 (0)	1 (100.0)	0 (0)		
	Lohmann	4 (66.7)		0 (0)	0 (0)	1 (16.7)		

In the above table (Table 22) there were four significant p-values for association tests between isolation rates of different pathogens and study variables; genetics/breed of the chickens (farm and pools of samples level), location (farm level), and sample matrix type investigated (pool-level). Actually, these p-values didn`t give a strong biological meaning as they were compared between and among unmatched categories. The matrix type comparison yielded a significant p-value as all the respiratory tract samples gave negative test results which significantly deviated from results of cloacal swab samples that gave a

strong positive result. The results concerning the breed/genetics and location-based regression analysis, yielded significant p-values due to the single broiler farm, having a different Cobb 500 breed of chickens and only located in Bishoftu and be the only farm tested and became positive for IBDV.

NDV was isolated from cloacal swab pool (a pool of 10 swabs) collected from Mojo site. The farm from which NDV was isolated was a small-scale poultry farm (flock size = 200) that had a one-year old Lohmann layers hens. The owner of the farm had reported the use of Newcastle disease vaccine (Lasota) administered at three, six and nine months of their age. There was no clinical sign of any poultry disease observed in the farm during the investigation period. There were no deaths reported, and the farm didn't use any antimicrobial drugs for those particular sample flocks in the farm. This farm was additionally tested positive for *Campylobacter jejuni* in addition to the NDV (Table 21).

IBV was isolated from two different farms, one layers farm and the other a broilers farm from Bishoftu. The layer farm was a medium-scale poultry farm (flock size = 3000), owing a 3 months old chick of Bovans brown breed. The birds in the farm were reported to be vaccinated for Newcastle disease on day 28, 35, and 40. On day 40 the chickens were vaccinated also for fowl pox and Marek's diseases. The layers farm from which IBV was isolated had reported that infectious bronchitis vaccine was not administered for the chickens since their introduction to the farm. The positive vaccinal strain-related IBV isolated in the study (4/91-like strain/793B) was more probably related to vaccine administered for the chickens while they were at their source farm. This was actually confirmed through cross-checking with the source farm. This farm has reported no antimicrobial drug use. A yellowish diarrhea with depression was observed in some of the sampled chickens of the farm, though these signs were not directly implicated to the IBV isolated. In this farm, IBV was isolated together with *Campylobacter jejuni* from the same sample pool of ten cloacal swabs, just like the case in the NDV positive farm and pool.

The second IBV positive farm was a broiler farm that was positive for IBDV as well. This farm was a large-scale broiler farm from which bursal and kidney tissue smears on FTA

cards were collected. Two kidney tissue smear pools from two different sheds of the farm were positive for IBV, while three pools of bursal tissue imprints, each from a different shed of the farm, were positive for IBDV (Table 21). This broiler farm was the one that has distributed chickens of different age for other farms on need base. This farm has layers bird farms at different site, and the layers farm that was positive for the first IBV isolate from cloacal swab pool had reported introducing the chickens from this farm. This large-scale broiler farm has reported to use a vaccination program for different viruses where on day 1: NCD vaccine, Gumboro vaccine, and IB vaccine; on day 7: NCD vaccine; and also, on day 16: NCD vaccine was administered. The breed of the chickens was Cobb 500 and their age ranged from 9-23 days. The farm has reported the use of Norfloxacin antimicrobial drug. Up on clinical examination, torticollis was seen in some of the chicks, and the presence of a similar sign was reported by farm attendants as being frequently manifested by different flocks of chickens so far hatched in the farm. Urates on kidney was also seen at post mortem examination in some sample moribund chicks during kidney tissue sampling.

IBDV was isolated from two sheds/flocks of this broiler farm, all from a different bursal tissue smear pool (Table 11). The bursal tissues of the moribund birds, up on postmortem examination, were found atrophied in some birds, and became swollen in others. The isolated IBDVs were phylogenetically analyzed and confirmed to be a vaccinal strain similar to the one reported to be used in the farm during the study period (with 99.8–100% identity to the Winterfield-2512 vaccine strain belonging to the classical/virulent genogroup A1a).

4.6. Analysis of Association between Campylobacter and Viral agent isolation rates

The regression analysis for the dependent type association between Campylobacter isolation rate and that of viral agents in the swab samples was conducted. Ten farms that were positive for Campylobacter were virus test negative, while two Campylobacter test positive farms were also positive for viral tests (Table 22). In other way, all the

Campylobacter test negative farms were also negative for virus isolation tests. However, the test for dependent type association between the Campylobacter and viral agent isolation rates showed a non-significant association ($p > 0.05$) between the two groups of pathogens of poultry.

Table 23. Campylobacter isolation rate Vs virus isolation

		Virus			
		Positive	negative	Total	p-value
Bacteria (farm)	positive	2 (16.7)	10 (83.3)	12	1.000
	negative	0 (0)	3 (100)	3	
Total		2 (13.3)	13 (86.7)	15	
Bacteria (pool)	positive	2 (14.3)	12 (85.7)	14	0.117
	negative	0 (0)	26 (100)	26	
Total		2 (5.0)	38 (95.0)	40	

There was no co-isolation detected in this study except in two farms, one farm found positive for *Campylobacter jejuni* and NDV (a farm in Mojo) and another one was for *Campylobacter jejuni* and IBV (a farm in Bishoftu). In fact, in both cases it was *C. jejuni* that was associated with the viral agents isolated. In the remaining of the cases, even though a pair of respiratory and cloacal swabs were purposely collected from each of the sample sheds to investigate if there is any association between bacteria and virus isolation, due to all the respiratory swab samples became negative, it became impossible to draw a valid conclusion about the association. At the same time, it became impossible to compute a valid statistical test about an association, particularly between Campylobacter, and NDV or IBV. It was a single cloacal swab pool for each of the viruses that was tested positive. Due to this low level of detection of the viral agents, a valid test could not be possible. The other thing is, since a different sample matrix were investigated for IBDV (bursal tissue FTA card) and Campylobacter spp. (cloacal swab) from different farms and different study subjects (dead birds, and live birds respectively), it was impossible to mention about their association. But, though a meaning full statistical test couldn't be computed, from the present test it can be seen that Campylobacter was detected from farms with and without

the other infectious agents tested. That is, Campylobacter isolation was neither limited nor enhanced by any specific risk factor investigated in this study.

4.7. Public Health Significance of the isolated agents

In this study, a major public health significant *C. jejuni* was predominantly isolated (71.4%,). This was 10/14 total pools positive for campylobacter which was 9/15, 60% farms. Being a major zoonotic pathogen, highly virulent, tetracycline-resistant, and having a wide-spread occurrence in the environment, makes *Campylobacter jejuni* a pathogen of public health importance. The present high rate of isolation of *Campylobacter jejuni* from sample chickens has a direct implication on its wider distribution among human population, particularly among those who are directly involved with animal handling and processing of foods of animal origin. The risk of acquiring this pathogen was further been exaggerated by the poor biosecurity practices performed in those sample poultry farms.

Most of the farm attendants encountered during the study period were non-professionals, teenagers who didn't have any experience in handling poultry either through life-long knowledge or specific training. On top of these, they were not provided with supplies like personal protective cloths; overalls, boots, gloves, and disinfectants, foot baths, and sanitary chemicals. Though these inputs were provided in some of the studied farms, the attendants were not properly using them and there was no enforcement in place to obliged the attendants and other workers to comply with the pre-sat standards.

As a result of these, there were high risk of pathogen entry to human subjects from animal reservoirs. These could happen either through direct contact with infected poultry, contaminated premises, and contaminated food, or consumption of contaminated food of poultry origin. From these primary infected human subjects, pathogens could also pass to other human and could also re-infect poultry in the same farm or other farms. The potential infection of other farms can be exemplified by the farm attendants looking after more than one farm being compounded by unsecure practices regarding biosecurity measures to be

followed. These could be one reason for the isolation of Campylobacter from most of the studied farms (70%) regardless of the farm sites, age and breed of the birds, flock size, and antibiotic use.

The wider distribution of Campylobacter among different poultry farms could further expose larger proportion of human being to the infection. This was particularly true in the commercial farms studied as they have distributed the life birds and poultry products (meat and eggs) to different towns and cities in Central Ethiopia. This uncontrolled movement of poultry and their products could potentially distributed Campylobacter and other pathogens along the distribution chain of poultry and their products. Being exacerbated by the poor bioexclusion and biocontainment measures in place, there were high risk of entry, passage and distribution of Campylobacter and the other pathogens of current concern among poultry and human population in the study area, and similar areas in the country.

NDV that has a known public health importance causing mainly conjunctivitis and also other health complications in human was also isolated in the present study. Infectious bursal disease virus (IBDV) was also among the viral isolates. It usually affects young chickens and weakens their immune system, predisposing the birds to vaccination failure and opportunistic pathogens that could be of zoonotic importance. IBDV does not have any zoonotic potential, but its immunosuppressive nature could favor the replication of pathogens of zoonotic importance, such as Salmonella spp., Campylobacter spp. and Avian influenza virus (Subler *et al*., 2006; Eterradossi and Saif, 2013). IBDV infection can exacerbate colonization and shedding of Campylobacter, particularly *C. jejuni*, likely through the immune suppression this virus causes in chickens. The disease seriousness relies upon the virulence of the strain and on the host's immune state (Subler *et al*., 2006). In conclusion, it can be said that there was high risk of pathogen transfer from chicken population to human population of the study area, complicating the public health status of the communities at large.

5. DISCUSSION

5.2. Discussion on Campylobacter and Salmonella

The study on pathogens of chickens with direct and indirect zoonotic importance was conducted with the aim to detect, identify and molecularly characterize major pathogens of chickens with public health significance. Based on these, appropriate samples were collected together with related field data, molecularly tested for isolation of the major bacterial and viral pathogens of chickens with public health importance, and finally association tests were conducted, and interpretation about the public health importance of the identified pathogens, were made to meet the objectives. Assessment of the public health importance of the isolated pathogens were conducted based on the isolation rates of the zoonotic pathogens as well as through assessment of the existence of potential risks of transmission of the identified zoonotic pathogens from chicken hosts in which they were detected to human population, particularly to those who were closely involved with management of those sample farms from which the positive isolates were identified.

Accordingly, different pathogens of chickens, belonged to both bacteria and virus, were identified in the study. The major bacteria isolated was Campylobacter while the viral pathogens isolated were NDV, IBDV, and IBV. Those detected pathogens were further identified to their species and strain-levels and found to be *C. jejuni*, *C. avium*, and *C. helveticus* for Campylobacter while all the three viruses detected were of vaccinal strains of their respective virus group.

In the current study, *Campylobacter* species were identified from most of the study farms (80.00%) regardless of the flock size, age of the birds, use of antimicrobial drugs and clinical sign presence. In line with the previously reported data in Ethiopia (Ewnetu and Mihret, 2010; Budge *et al*., 2020), the findings of the present study assured that campylobacter can be isolated from poultry under any situation as *Campylobacter* spp. is a commensal bacterium in poultry, where it was commonly isolated from both egg-type and meat-type chickens (Brena *et al*., 2016). It was *C. jejuni* species which was most

frequently detected in most studies of the type. The identification of the two other species detected in the present study is more interesting: in fact, there is no previous reports of *C. avium* and *C. helveticus* in poultry in Ethiopia. *C. helveticus* was actually considered a risk related particularly to cats and dogs, which generally act as reservoirs of subclinical infection (Threlfall, 2002). However, the scarce data about the circulation of these species should be imputed to the lack of diagnostic surveys rather than to their absence in the field, highlighting the usefulness of the present study.

On the other hand, all sample farms in the present study were tested negative for *Salmonella* spp. despite the previous detections in poultry farms in central Ethiopia (Eguale, 2018). Absenteeism of a single isolation of salmonella, in spite of the isolation of the other test pathogens was a question, but it could happen as a result of many factors. One possible reason could be absence of a single pathogen in the samples collected. The other reason could be the sample type used. Salmonella PCR is usually used for environmental surveillance testing. But if a Salmonella PCR is requested on an enteric sample, both a bacterial culture and PCR are required to increase the testing sensitivity.

A decrease in the susceptibility of *Salmonella* spp. was indicated for some of the antimicrobial drugs (Abdi *et al*., 2017) (oxytetracycline, sulfadiazine, norfloxacin) that were reportedly administered in the farms enrolled in the study. This finding is not representative of the actual epidemiological situation of *Salmonella* spp., since the diagnostic method and the sampling type (dry swabs instead of enriched transport medium) used in this study are not the gold standard. The tests were performed on the same samples which were positive for *Campylobacter* spp., thus ruling out problems during the extraction phase or related to the conservation of the nucleic acids. However, it cannot be excluded that the bacterial titer in the samples could have been under the limit of detection of the biomolecular assay or that the intermittent shedding of *Salmonella* spp. could have led to negative results due to the single sampling in the farms. Environmental sampling or a larger bird sample size should be preferred when investigating *Salmonella* spp. Still, different isolation rates of *Salmonella* spp. are reported in Ethiopia, ranging from 4% to 16% (Assefa *et al*., 2011; Eguale *et al*., 2015), suggesting a certain variability in the prevalence.

Differently from *Salmonella* spp., detected in egg content in an Ethiopian study (Mintesnot *et al.*, 2011), egg consumption is not one of the main routes of human infection with *Campylobacter* spp. (McEwen and Collignon, 2018). Nevertheless, its high prevalence in the farm could definitely lead to shell contamination, thus increasing the risk of ingestion, surface contamination and passage through the shell during handling or storage.

Unfortunately, the biomolecular nature of the survey did not allow us to further explore the patterns of antimicrobial susceptibility of the detected bacteria, especially in the farms where the use of antimicrobials was recorded. However, the wide antimicrobial use and the increasing spread of *Salmonella* spp. and *Campylobacter* spp. antimicrobial-resistant strains (Kramer *et al*., 2000) highlight the importance of a continuous epidemiological update on these micro-organisms, resorting to different diagnostic approaches.

It was reported that, in Ethiopia, antimicrobial drugs can sometimes be administered in the absence of a clear diagnosis or susceptibility testing in response to various clinical signs or alterations (Assefa *et al*., 2011). Even if unrelated to the investigated pathogens, clinical signs have also been recorded in this study, attesting conditions where management could have been suboptimal. In fact, some of the farms involved in the present study reported clinical problems and the use of oxytetracycline, sulfadiazine and norfloxacin without disclosing the therapeutic indications and previous diagnostic tests.

In this study, when more than one shed in the farms was sampled, different scenarios were displayed, even in light of the same farming conditions: in two farms, one shed was positive for *C. jejuni*, and the other one was negative, whereas two other farms revealed the presence of different species of *Campylobacter* in different flocks. This finding is not unexpected since the coexistence of multiple strains was reported (Gölz *et al*., 2014) and, in case of inadequate biosecurity measures, poor disinfection between cycles or contamination of the birds at the source, different bacteria could persist or be introduced in the same environment. Moreover, common biomolecular and Sanger-sequencing methods yield only single information about the detected species, generally revealing the most abundant

species but potentially masking co-infections sustained by genetically similar pathogens. However, the quality of the sequences obtained in the present study supports the herein identified species. Despite this limitation, the presence of different species in the same farm was detected, revealing the heterogeneity of the circulating *Campylobacter* species.

Since the prevalence of *Campylobacter* spp. in poultry is determined by a wide range of factors, such as the contamination of the birds at the hatchery, life length, egg or meat production, farm and flock size, seasonality, litter, water, feed and management of environmental conditions, biosecurity might not be enough to avert flock colonization (Carrasco *et al*., 2012). However, good hygiene and manufacturing practices should not be restricted to the farming level, and Campylobacteriosis risk can be contained along the food production chain by limiting cross-contamination at slaughter and implementing strict disinfection procedures thus lowering the bacterial load. Regular monitoring on farms could also help to identify contaminated flocks that are destined for products restricted to cooking by thorough heat treatment for microbial inactivation (Sampers *et al*., 2010). The strict implementation of a cold chain during meat processing and distribution is another pivotal aspect for reducing microbial load on meat and preventing cross-contamination of other food products, tools and surfaces (Sampers *et al*., 2010). Still, this does not prevent contamination related to handling procedures at the farm level, where workers might be exposed to and carry *Campylobacter* spp. and *Salmonella* spp. if not well trained towards safety and good hygiene practices.

5.2. Discussion on Viral Agents Detection and Identification

All the respiratory tract swab pools tested for viral pathogens, IBV, NDV, and aMPV were negative. Though the test has targeted for pathogens that can probably be found in respiratory system based on their tropism, all of the twenty swab pools tested became test negative for the pathogens. This could probably be due to either the absence of the pathogens at the collection time or in the specific samples taken, or problem with the collection, storage, transportation, and PCR tests. These logics are based on indicators like isolation of vaccinal strains of NDV, and IBV from cloacal swabs of two different farms in the study, from which the negative test result has be encountered for their respective respiratory swab pools. Generally, the low overall isolation rates of viruses in this study

suggested a low circulation of the pathogens, probably owing to vaccination strategies or problem with the sample collection, transportation, and laboratory analysis.

Isolation of the vaccinal strains from cloaca swab samples, together with the information obtained from field data, further indicated the wider use of vaccine which can attributed to negativity of the tests conducted on respiratory tract swab pools and low detection level among cloaca swab pools. Contrary to this, there were signs of disease, particularly of Newcastle disease, and at the same time farm attendants and owners have reported the same problem with Newcastle disease outbreak, complaining for the effectiveness of the vaccine they were using, LASOTA. The observed clinical sign of Newcastle disease, and the reported problem of Newcastle disease outbreak, may be due to either the occurrence of a field strain which can't be detected in this study or may be due to seroconversion of the vaccinal strain used.

In the present study, detection of the investigated viral pathogens was limited to three farms only: each farm was positive for a different agent except IBV and IBDV (two farms for IBV, one for NDV and one for IBDV). Though it became impossible to further characterize two of the IBV detections from the same farm, the rest characterized strains appeared to be vaccine strains (a 4/91-like vaccine strain, a Lasota-like vaccine strain and a Winterfield-2512-like vaccine strain), indicating either the persistence of the administered vaccine or the spread of vaccine strains from neighboring farms. The positive samples were collected from layer and broiler birds that were reported to be vaccinated against different viral diseases, including those investigated here (IBV, NDV and IBDV), with vaccines based on the detected strains for IBDV and NDV. Regarding IBV detection, the introduction of a vaccine-derived strain from an unknown source (farms implementing a different vaccine protocol, contaminated fomites or personnel) cannot be excluded, since the protocol that was applied on the farm involved different strains. The persistence of a vaccine strain is a common finding when live vaccines are administered, because they can be shed in feces, followed by re-uptake by the birds and subsequent collection during sampling, thus complicating the diagnostic process. However, the within-flock circulation of live vaccines can also lead to vaccine reactions, when the initial coverage for the birds is only partial

(Jackwood and Lee, 2017). This aspect does not really explain the clinical signs recorded on the positive farms, since different clinical signs (enteric signs, data not shown) to those from typical vaccine reactions were reported on the farm where an IBV vaccine-like strain was detected. Furthermore, on the farm where an NDV vaccine-like strain was detected, no clinical signs were registered, which is desirable. But on different farms covered in the study, a typical ND clinical signs were encountered even though the molecular tests yielded negative results. On the broiler farm where IBDV vaccine-like strains were detected, the main recorded lesions were urate deposits in the kidneys. Even though, in some cases, the reported clinical signs might have been partially suggestive of the investigated pathogens, the actual cause should be ascribed to other problems, most likely of both infectious and managerial origin. The absence of field strains is surprising, given that the local epidemiology and previous work reported the consistent presence of pathogens such as NDV (Chaka *et al*., 2012; Terefe *et al*., 2015), IBV (Hutton *et al*., 2017; Tegegne *et al*., 2020) and IBDV (Negash *et al*., 2012; Lemma *et al*., 2015). This finding is also supported by the low mortality rates reported in some cases by the farmers. A certain seasonality, with a higher occurrence of NDV outbreaks during the pre-rainy season, was proposed (Haile and Fentie, 2020), and the timing of sampling (March) could have influenced the detection rate in the present study. Conversely, all farms declared that they vaccinate their birds, although they did not disclose the complete protocol. The implementation of vaccination surely plays a role in preventing viral circulation, together with possible previous natural infection, and this could have contributed to the acquisition of natural immunity, which was not investigated by serological means in this study. Vaccination in Ethiopia is often performed on the farm, at the hatchery or at the source, before introducing the animals to the farm, usually starting from one day of age (Habte *et al*., 2017). In fact, Oromia, the region where the study was set, is one of the regions in Ethiopia with the highest accessibility to vaccination and veterinary services, as reported by Asfaw *et al*., (2021). Vaccination and biosecurity are the key factors in achieving disease control and efficient production, but these measures are often difficult to apply to rural or village farms. However, in this study, small-sized farms (<500 birds) were also free from field strains, suggesting the presence of fair biosecurity levels, prophylactic measures and limited contact with neighboring farms or other potential sources of infection.

6. SUMMARY

6.1. Conclusions

High rate of isolation of campylobacter was encountered in the study area. Three species of campylobacter, namely C. jejuni, C. avium and C. helveticus were detected. The detection of Campylobacter bacteria, particularly the public health significant *C. jejuni* species, suggested a wide spread occurrence of this pathogen among poultry farms and households that kept poultry in Ethiopia. This further imply its greater risk of introduction to human population, specially, to those who are occupationally involved with poultry. The risk of introduction of this agent to human population will be doubled, or tripled, being compounded with poor biosecurity practices, and poor awareness about zoonotic diseases in the country. Herein lies the importance of molecular surveys in developing countries, such as Ethiopia, where intense diagnostic monitoring should be performed to support the growing poultry sector and to control the associated increased disease risks both in poultry and human population. The present study evidenced the presence and heterogeneity of different species of *Campylobacter*, drawing attention to the implementation of biosecurity measures, good hygiene practices and animal management. Conversely, the lack of *Salmonella* spp. and aMPV detection and low level of detection of viral agents (NDV, IBDV and IBV) suggests the importance of dedicated studies, employing classical molecular approaches that would also allow the evaluation of the virulence profile of the detected pathogens.

The low circulation of these viruses in this region limits the risks associated to their role as door openers for secondary pathogens, impacting not only poultry production but also public health. But, even this limited isolation of the viruses, particularly NDV, and IBDV, suggested their greater impact to poultry sector and public health, owing to their virulence and public health consequences. NDV can directly impact public health causing eye infection and upper respiratory problems if introduced to human population. Though all viruses are known by their immunosuppression, IBDV is well pronounced by its immunosuppression and exposing to secondary bacterial infections like

Campylobacteriosis. Particularly, IBDV infection exacerbated colonization and shedding of *C. jejuni*, presumably through the immune suppression this virus causes in chickens.

Thus, in conclusion, this study displays a reassuring picture of the epidemiological situation in the study area, Bishoftu and Mojo, central Ethiopia, and aims to stress the importance of thorough monitoring, information sharing and the implementation of both vaccination strategies and biosecurity measures.

Limitation of the study

- Sample size: small sample size is not a good representative of a study population. As the sample size increases, approaching the population size, the sample will be a best representative of the population. In this study, though the total sample unit (chickens) sampled are not small, pooling their samples in pools of ten chickens in a single shed, made the sample analyzed small. Since the result was analyzed based on pools of samples as a single study unit, the result didn't allow full interpretation of the epidemiological analysis conducted.
- Location being specific to small area coverage: this study was limited to two towns with potential poultry productions among areas in central part of the country. Wider area coverage in a study will let comparisons in results from different areas. But wider area coverage in a study, has implicated additional costs beyond what can be afforded.
- Lack of Microbiological works: some molecular tests demanded a microbiological culture preceding or accompanying it. This study was purely depended on molecular test based on nucleic acid extraction from field sample without culturing. This might limit the result on field pathogens.
- Lack of corresponding human samples that can complement results and discussions on public health importance of the identified pathogens from poultry samples.

6.2. Recommendation

- Poultry disease agents' control scheme should be in place, particularly, campylobacter control should get attention, as results from the present study has showed high prevalence of the pathogen in the field indicating lesser control measures directed against it.
- There should be a regular investigation for detection of existence of pathogens circulating in a field, and to detect emergence of new pathogens, or new types or strains of the existing pathogens.
- Reporting of some Campylobacter species for first time in Ethiopia indicated a need for nationwide and regular surveillance to monitor existence of undetected species, subspecies or strains of pathogens
- An extensive diagnostic approach with enough investments should be implemented to detect important pathogens of poultry that were clinically examined during field data collection in this study and also reported by poultry farm owners, and attendants of the sample farms, but fail to be detected or detected only in low level in the study, against the facts on the ground
- The usefulness of some vaccination, particularly of the Newcastle disease, should be double checked as there were encounters for clinical diseases of New castle and reports from concerned individuals showed for an outbreak of the disease against an extensive use of New castle disease vaccine (Lasota), might be due to vaccine reversion or vaccine failure

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8. APPENDICES

Appendix Table 1: Sample collection templets for information to be filled individually for each sample farms

Name, address and contacts									
Sample ID-Code									
Matrix (oropharyngeal, tracheal, cloacal swabs, FTA card, bursa)									
Collection date									
Farm / Shed / Flock									
Location (Address, City, Country)									
Origin of the chicks									
N° of sheds / N° of birds									
Species									
Production type / Sex (Broilers, Breeders, Layers)									
Genetic type (e.g. ROSS, Cobb, local breed)									
Age at sampling									
Vaccination protocol	**Age**	**Vaccination**	**Route**	**Age**	**Vaccination**	**Route**	**Age**	**Vaccination**	**Route**
Contact with other farms (trucks, Feed mill trucks, sharing equipment and personnel, others…)									
Integrated company or Owner? Please indicate also the name									
Clinical signs									
Lesions									
Morbidity									
Mortality									
Zootechnical parameters (e.g. mean weight, f.c.r)									
Treatment applied (i.e. antibiotics)									

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