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Biosecurity Practices and Molecular Epidemiology of *Salmonella Species* in Small and Medium-Scale Commercial Layer Farms in Mekelle City, Northern Ethiopia

By

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ADVISORS APPROVAL SHEET

This is to certify that the thesis entitled **"Biosecurity Practices and Molecular Epidemiology of Salmonella Species in Small and Medium-Scale Commercial Layer Farms in Mekelle City, Northern Ethiopia"** submitted in partial fulfillment of master of science (MSc) in Tropical Veterinary medicine, the Graduate program of Mekelle University, College of Veterinary Sciences has been carried out by **Aregawi Gerekidan Abera (**ID .NO. cvm/ pr/010/13) under our supervision. Therefore, we recommended that the student has fulfilled the requirements and hence hereby can submit the thesis to the department.

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AUTHOR DECLARATION

I declare that this thesis presents the work carried out by myself and does not incorporate without the acknowledgement of any material previously submitted for a degree or diploma in any university; and to the best of my understanding, it does not contain any materials previously published or written by another person except where due reference is made in the text; all substantive contributions by others to the work presented including jointly authored publications, is clearly acknowledged.

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ACRONYMS AND ABBREVIATIONS

AGIDT	Agare Gel Immuno Diffusion Test
BGA	Brilliant Green Agar
BPW	Buffered Peptone Water
CSA	Central for Statistical Agency
CDC	Centre for Disease Control
CPCR	Conventional Polymerase Chain Reaction
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EB	Elution Buffer
ISO	International Organization for Standardization
NTS	None Typhoidal Salmonella
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
RVS	Rappaport Vassiliadis
TAE	Tris-Acetate EDTA
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

ABSRTACT

Salmonellosis infections in chicken farms pose significant risks to public health and the poultry industry. In the Tigray region, there is a notable lack of molecular-based identification methods for Salmonella species in poultry and limited understanding of existing biosecurity practices. This study aimed to isolate and molecularly characterize Salmonella species while evaluating the biosecurity measures practiced on the farms. A cross-sectional study was conducted between December 2023 and December 2024, involving the collection of 250 cloacal swabs from laying hens and identifying biosecurity practices across 28 purposively selected poultry farms. Salmonella species were isolated following the International Organization for Standardization's (ISO 6579-1:2017/Amd.1:2020(E)). Out of 28 poultry farms tested using standard bacteriological culture methods, 16 (57.1%) were positive for Salmonella isolates. The overall isolation rate of Salmonella species was 34.8% (87/250). Among the analyzed risk factors, on farm waste disposal practice, sources of chicken, presence of other animals, age and flock size demonstrated a statistically significant association ($p < 0.05$) with bacteriological rate of isolation Salmonella species. The molecular detection rate of Salmonella genus was 82.4% (42 out of 51) successfully amplified with the INVA1/2 primers. Notably, 45% of these isolates were identified as Salmonella gallinarum using MS-GA-F/R primers, while 35% were identified as Salmonella typhimurium with TYPHF/R primers. The poultry farm biosecurity parameters and practices were evaluated by a scientific risk-based scoring system based on the Biocheck. UGent™ tool (Merelbeke, Ghent University, Belgium) (https://biocheck.ugent.be/en). The overall biosecurity score for layer farms in Mekelle is 57.86%, with the scores of 48.54% for external biosecurity and 67.11% for internal biosecurity. Adapting to evolving biosecurity challenges requires the adoption of new toolkits and strategies. It showed the biosecurity measures of these farms, especially at the external levels need to be improved. The investigation revealed a higher isolation and identification rate of *Salmonella species* at both the farm and sample levels. Therefore, routine flock testing, farm surveillance, implementation of Salmonella prevention and control strategies, and improvements in biosecurity practices is needed.

Keywords: Biosecurity, Isolation, PCR, poultry farms, *Salmonella*, Mekelle

1. INTRODUCTION

1.1 Background

Poultry products are the most important sources of protein for all populations, and in some countries, they are also thought to be the most beneficial for the economy and income (Attia *et al.,* 2022). Programs aimed at reducing poverty in developing nations like Ethiopia greatly benefit from the production of poultry. It gives impoverished rural communities access to quick cash, creates jobs, increases family food security, and strengthens the position of women (Mossie, 2018). But a lot of things prevent chicken industry from operating at its best. These include infectious diseases including salmonellosis, mycoplasmosis, infectious bursa disease, Newcastle disease, and others (Chaka *e t al.,* 2012).

One of the most significant and potentially fatal bacterial zoonotic diseases that affects both humans and animals is Salmonellosis (Abd El-Ghany, 2020). Salmonella has been found to be the major cause of food-borne diseases and a serious public health problem in the world, with an increasing concern for the emergence and spread of antimicrobial-resistant strains (Ejo *et al.,* 2016). *Salmonella species* belong to Gram negative, rod shaped, and facultative intracellular bacteria that potentially infect a wide variety of hosts. Salmonella is comprised of two species, *Salmonella bongori* and *Salmonella enterica* (Chen *et al.,* 2021). Most infections are due to the ingestion of foods contaminated by animal feces, or by environmental pollutions. Salmonella is the most common bacterial pathogen causing gastrointestinal infection worldwide(Pires *et al.,* 2021, Lee and Yoon, 2021) .

In Ethiopia, infectious diseases like Newcastle disease, Salmonellosis, Coccidiosis, fowl cholera, and fowl pox are the primary causes of high morbidity and mortality in chicken production at all scales (Asfaw *et al*., 2021). One of the most significant bacterial diseases affecting the poultry industry and other bird species is Salmonellosis.

It reduces productivity, leading to significant economic losses, and poses a public health risk due to zoonotic transmission, which can be costly to treat(Shekhar, 2018) . In Ethiopia, the prevalence of Salmonella species in poultry has been a growing concern, with various studies indicating significant rates of infection across different regions and farming practices. The farm-level prevalence of Salmonella varies from the highest at

73.07% in and around Debire Birhan, 50.6% in Central Ethiopia, 36.2%, in Addis Ababa, and 14.6% in Central Ethiopia (Ayele, 2021; Eguale, 2018; Akalu *et al*., 2024; Waktole *et al*., 2024). Furthermore, sample-level prevalence was high respectively in and around Debire Birhan (14.06%), Addis Ababa (6.4%), across Central Ethiopia (14.4 %.), central Oromia (15.12%), and in and around Arba Minch town, Gamo Zone (5.8%) (Ayele, 2021; Akalu *et al.,* 2024; Waktole *et al.,* 2024; Abunna, 2018; Abayneh *et al.,* 2023). Several Salmonella serovars have been identified across in Ethiopia and have been isolated from people, animals, food containing animal products, and their surroundings (Dagnew *et al*., 2020).

A number of interrelated risk factors, such as flock size, chicken source, housing systems, and management techniques, affect the prevalence of Salmonella in poultry farms. Purchasing chickens from several breeding facilities can transmit Salmonella, particularly if those facilities don't have stringent biosecurity protocols, and larger flocks are more susceptible to outbreaks because of the increased bird density. If not properly maintained, housing systems-especially floor housing-may produce conditions that encourage the growth of bacteria. Excrement-contaminated feed poses a serious risk of transmitting Salmonella, and improper on-farm waste disposal can contaminate the environment. Farms that disregard cleaning procedures are more susceptible to epidemics; regular disinfection procedures are crucial. Contact with nearby farms can raise the risk of contracting Salmonella, and farm workers' age and experience can affect their adherence to biosecurity protocols. Reducing the prevalence of Salmonella and guaranteeing healthier flocks and safer food products for customers depend on addressing these variables (Abdi *et a*l., 2017: Eguale, 2018: Dagnew *et al*., 2020: Akalu *et al*., 2024: Samper-Cativiela *et al*., 2023: Abayneh *et al.*, 2023).

"Biosecurity" refers to a broad range of measures intended to lower the risk of introducing and dispersing diseases (Alarcon *et al*., 2021). In layer farms, any intervention to improve adherence to biosecurity measures remains the most practical and economical way to eradicate disease (Dewulf, *et al*., 2019). A robust biosecurity plan is essential during disasters to prevent the spread of infectious diseases among animals and from animals to humans. Effective biosecurity practices can significantly reduce the risk of zoonotic transmission(Thakur, 2022). It is well established that effective biosecurity

measures at the farm level are essential for safeguarding the health of poultry and ensuring the safety of animal-derived products throughout the entire poultry production value chain. By implementing robust biosecurity practices, farmers can significantly reduce the risk of disease outbreaks (Gelaude *et al.,* 2014).

Biosecurity at a poultry farm includes all measures taken to minimize the risk of introduction and spread of disease agents and thus, includes all actions for keeping poultry and the farm healthy. By taking these biosecurity measures and performing efficient management, on-farm animals are protected against both endemic and epidemic diseases (Maertens *et al*., 2018). Biosecurity techniques are crucial to preserving and enhancing animal health while lowering hazards. The dangers of infection introduction (Manuja *et al.*, 2014). Biosecurity lowers the risk of contracting a disease, raises welfare and production, lowers downgrades, and raises profit. Additionally, biosecurity lessens the chance of rare viruses like avian influenza, which may possibly destroy a company and endanger the entire industry (Otte *et al*., 2021).

The practice of biosecurity measures in Ethiopia's poultry sector has been critically assessed in several studies, revealing significant shortcomings. The study titled "Quantitative Assessment of Major Biosecurity Challenges of Poultry Production in Central Ethiopia" found an alarming overall biosecurity score of only 43.1% among poultry farms in the region. This score is notably lower than the global average of 64.3%, highlighting substantial deficiencies in biosecurity practices (Waktole *et a*l., 2023). In a related study conducted in Bishoftu, it was emphasized that there is a pressing need for improved biosecurity practices across commercial chicken farms (Ismael, 2021).

Further research conducted in the Adama, Ada'a, and Lume districts corroborated these findings, revealing that a significant majority of farms also exhibited poor biosecurity practices,with Only 23.5% of the farms surveyed achieved a good biosecurity score, while a concerning 76.5% had poor scores (Tadesse *et a*l., 2024).

A few researches were conducted ten years ago to detect the presence and evaluate the prevalence salmonellosis infection within a flock based on serological tests. As a results they have reported varying prevalence rates of Salmonella in poultry (Berhe *et al*., 2012: Ashwani *etal.*,2014). However, there has been no published research on the molecular

isolation, identification, and characterization of Salmonella in small and medium-scale commercial layer farms in Mekelle City.

The Biocheck.UGent scoring system is a scientific, risk-based, and independent tool designed to evaluate the quality of on-farm biosecurity measures. However, information regarding the implementation levels of biosecurity practices in small- and medium-scale commercial laying hen farms in Mekelle City remains limited. This gap exists largely because of the absence of comprehensive research conducted in Tigray region to assess these practices using the Bio check UGent score system.

The study aimed to evaluate the prevalence of Salmonella species in small and medium-scale commercial laying hen farms in Mekelle city while adapting the new developed biosecurity scoring system developed by Bio check UGent score system. This scoring system is designed to provide a standardized and reproducible method for assessing biosecurity levels on farms, establishing clear benchmarks that will help producers maintain optimal biosecurity standards. By enhancing biosecurity, the system seeks to improve the health and productivity of laying hen flocks, ensuring better outcomes for small and medium-scale producers.

1.2 Objective of the Study

1.2.1 General Objective

This research aimed to isolate and conduct molecular characterization of Salmonella species, as well as to assess biosecurity practices in small- and medium-scale commercial laying hen farms in Mekelle city, Ethiopia.

1.2.2. Specific Objectives

- to isolate and characterize Salmonella species using Polymerase Chain Reaction (PCR)
- To identify risk factors linked to the occurrence of Salmonellosis in the farms.
- To identify the biosecurity practices in layer farms using the Biocheck.UGent scoring system.

2. LITRATURE REVIEW
2.1 Epidemiology of Salmonella species

2.1.1. Biochemical Characteristics

Salmonella are 0.7-1.5 x 2.5 µm, gram-negative, aerobic or facultative anaerobic, urea negative, oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmons citrate positive, and have the ability to produce H_2S. With the exception of *Salmonella pullorum* and *Salmonella gallinarum*, which are nonmotile, they are typically motile with peritricheal flagella and ferment carbohydrates with gas generation. They are also nonsporogenic (Wierup *et al.*, 2017). Around 7.0 is the ideal pH for multiplication; pH values of 9.0 or lower are bactericidal. The ideal temperature range is between 35 and 37°C, with a minimum of 5°C and a maximum of 47°C. Salmonella cannot survive concentrations of salt higher than 9%. It shares a close relationship with the genus *Escherichia* and can be found on every continent in both warm-blooded and cold-blooded animals as well as in nonliving environments (Yan *et al*., 2021).

Salmonellae is mainly found in the intestines of humans and animals, especially poultry and swine. They are excreted in feces and can be spread by insects and other means to various places such as water, soil, and kitchen surfaces. Salmonella infection mainly occurs through eggs, poultry, and raw meat products, with *S. typhimurium* and *S. Enteritidis* being the most commonly found (Eng *et al*., 2015). Some serovars (serotypes) have a habitat limited to a host species, such as humans (*serovars Typhi, Paratyphi A*), sheep (*serovar Abortusovis*) or fowl (*Gallinarum*) (Akpabio, 2015).

*2.1.*2. Global Distribution of Salmonellosis

Salmonella bacteria are known to be primarily found in the intestines of animals, but they can be found in various environments. Due to their ability to survive and adjust to challenging conditions, they are commonly found throughout the ecosystem (Marquez, 2023). They are commonly found in farm effluents, human sewage, and any substance that has been contaminated with fecal matter. Proper sanitary practices and improved hygiene can significantly reduce the spread of these bacteria. It is essential to properly

dispose of waste materials and practice good hand hygiene to prevent its transmission (Wray, 2003).

Salmonella is distributed worldwide and is endemic to areas where animal husbandry is practiced. Serovars also vary in their distribution across the world, with *S. typhimurium* and *S. Enteritidis* being prevalent everywhere. Some serovars are host-specific, like *Salmonella ser. abortusovis* in sheep, *Salmonella ser. choleraesuis* in pigs, and *Salmonella ser. dublin* in cattle. Typhoidal *Salmonella serovars like S. typhi and S. paratyphi* are human pathogens transmitted via the fecal-oral route. In contrast, NTS is zoonotic and can infect a wide range of animal reservoirs, including birds, reptiles, dogs, cats, and rodents (Syamily Shaji *et al.*, 2023).

2.1.3. Host range

Salmonella have a wide variety of domestic and wild animal hosts (Acha, 2001). All members of the genus are considered to be potentially pathogenic, although serovars may differ widely in their host range and the pathogenic syndromes that they produce. Some serovars appear to show a degree of host adaptation and primarily infect one animal species. They also tend to cause more severe illness than the other serovars (Foley *et al*., 2013) . Pullorum disease is a host-specific illness that primarily affects chicks under three weeks of age. The first signs of the illness are typically an excessive number of dead in-shell chicks and deaths soon after hatching, while it can also occasionally affect adult birds (Shivaprasad, 2000).

2.1.4. Morbidity and Mortality

Salmonellosis has a significant global economic impact and leads to serious illness and death in both humans and animals (Teklemariam *et al*., 2023). Outbreaks of Pullorum illness have occasionally been documented in older animals, but the disease is typically manifested in young birds. Age, bird strain, management, nutritional status, exposure route and dose, and additional disease stressors like concurrent illnesses in the flock all affect how many birds become infected (morbidity) and how many birds die (mortality) (Shivaprasad, 2000).

Morbidity and mortality in hens were significantly linked to their age. Research showed that older hens experienced higher levels of sickness and death compared to younger ones. This could be due to the fact that mature layers are more susceptible to the adverse effects of egg laying, leading to excessive strain on the birds and compromised immune defenses (Gedeno *et al*., 2022).

2.1.5. *Sources of infection and transmission*

Salmonella species are mainly transmitted through the fecal-oral route. They are then carried latently in the mesenteric lymph nodes and shed continuously or intermittently in the feces. While these bacteria are not actively shed, they can reactivate in response to stress or immune suppression (Hendriksen *et al*., 2004). The main source of infection for humans is poultry products (meat and eggs), which are frequently derived from healthy animals. There are many routes through which Salmonella may be introduced into a group of animals. These include animal feed, the environment, purchased and other animals that could get access to the farms (White, 2001). In dry areas, Salmonella is widespread and very resilient; it can endure in water for days or even months. Various hosts and reservoirs can contain different *S. enterica* serovars, which can affect both people and animals. Most *S. enterica* serovars are adapted to specific hosts, allowing them to infect and cause illness in various hosts, except for a few serovars restricted to specific hosts (Zamora-Sanabria and Alvarado, 2017).

These carriers play a crucial role in the ongoing spread of Salmonella in farms and the surrounding environment because they are able to excrete the bacterium regularly and sporadically in their feces without displaying any symptoms. Pets, including dogs and cats, have also been demonstrated to harbor the organism asymptomatically. As a result, they pose a risk to public health and other animals that produce food by periodically excreting the bacteria in their feces (Watson, 2024).

2.1.5.1. *Vertical Transmission*

Salmonella pullorum can contaminate the yolk, albumin, eggshell membranes, or eggshells before oviposition, leading to vertical transmission to the reproductive organs of hens. This contamination is a significant route for the pathogen to infect embryos and

chicks (Cui *et al.,* 2023). The presence of Salmonella pullorum in the ovules prior to ovulation can cause embryonic death and high mortality rates in chicks. This outcome highlights the pathogen's role as a primary mode of transmission within poultry populations (Haider *et al*., 2014).

2.1.5.2. Horizontal Transmission

Salmonella can move horizontally both inside and between flocks. Direct bird-to-bird contact, ingesting contaminated excrement, litter, food, drink, or people, equipment, and surroundings are some of the processes that mediate it. Additionally, pecking in contaminated soil or litter, eating sick eggs, wounds on the skin, and cannibalism of infected birds can all result in transmission within a flock. Positive identification of the egg as the primary mode of transmission and a significant contributor to both isolated cases and epidemics has been made (Wales and Davies, 2020).

2.1.6. Salmonella in Ethiopia

Salmonella was detected in chicken farms in Addis Ababa in 11.5% of cases. The probability that this result could lead to foodborne salmonellosis and pose a risk to human health via the food chain is significant (Mohammed and Dubie, 2022). In Central Ethiopia's poultry farms, 14.6% of the total bird droppings tested positive for Salmonella (Eguale, 2018). Based on a study in Southern Ethiopia, 16.7% of samples from three farms investigated were found to contain Salmonella (Abdi *et al.,* 2017). Another study discovered that 15% of poultry farms were found to be contaminated with Salmonella, whereas 8% of eggs sold in retail were infected (Assefa *et al*., 2011).

In Ethiopia, the habit of raw meat consumption and the presence of *Salmonella* in minced beef indicate, in addition to the poor hygienic standards in food handling in the country, the presence of great public health hazards of *Salmonella* (Loha, 2023). Among the characterized strains, Salmonella in Ethiopia, group A, B, D1, D2, and C were identified with varying frequencies. Specifically, 6 (15%) strains belonged to group A (Somatic antigen O, O:2), while 5 (12.5%) strains each were identified for groups B (O: 4), D1 (O:9), and D2 (O:9,46), and 3 (7.5%) strains were categorized under group C (O:7/O8) (Mengistu, 2011).

2.1.7. Salmonella in Tigray

The high seroprevalence of *S. galinarum* and *S. pullorum* in Tigray indicates that there is a significant presence of these infectious bacteria in the poultry population in the region. The prevalence of *Salmonella species* varies from 32.8% and 19.71% study by Berhe *et al.*, 2012 and Ashwani *et al*., 2014 respectively. Berhe *et al*. (2012) reported a prevalence of *Salmonella pullorum* in the vicinity of Mekelle to be 32.8%. The methodology utilized in the research was the slide agglutination test, which employed colored antigen of *Salmonella Gallinarum/pullorum*. A study utilized a plate agglutination test with a colored antigen of *Salmonella gallinarum* to screen serum samples from poultry in Mekelle and nearby areas (Adigrat, Hagersalem, Wukro), along with Debrezeit (Addis Ababa). The microagglutination test confirmed positive results with a prevalence of 19.71% by titrating the agglutination antigen of *Salmonella species*. Subsequent to confirmation testing by microagglutination with plain antigen of *S. gallinarum*, only 55 (19.71%) samples out of the plate-positive samples were found to be serologically positive, thus reducing the prevalence rate from 44.8% to 19.71%. This study highlighted significance of employing a specific confirmation test following initial screening for *S. gallinarum* infection through plate agglutination in order to obtain an accurate estimate of prevalence in poultry populations (Ashwani *et al*., 2014).

2.2. Risk Factors for *Salmonella* infection

Foodborne infections are mainly caused by various factors such as immunity, genetics, malnutrition, and socioeconomic status. Both the environment and the host can influence these risk factors (Amenu, 2013). Several factors impact the seriousness of a Salmonella infection, such as the host's age, immunity, presence of confections, environmental stress levels, management characteristics, and the infectious dose. For instance, even at 106 CFU/mL of *S. typhimurium*, older birds are frequently less susceptible to Salmonellosis (Hidayatullah *et al*., 2020).

2.2.1 Animal risk factors

This disease results in high mortality rates, which can approach 100%, a drop in egg and chick production, the condemnation of the afflicted carcass, and expensive medical expenses for both humans and animals. These factors together account for significant economic losses. Estimates are produced as part of a multidisciplinary considerations that includes direct health expenditures including hospitalization, doctor consultations, laboratory tests, and lost labor related to a salmonellosis (Tadele *et al*., 2014). The mortality rate is highest in newly hatched chicks, and the capacity of infection to survive rises with age. Because *S. pullorum* antibody is more common in adult poultry and pullorum disease is typically diagnosed in chicks younger than three weeks old, it is difficult to obtain the antibody on these birds unless they survive and become carriers. As a result, seroprevalence increased with the age of the birds and there was a high significance difference (Netsant *et al.,* 2012). Animal response to *Salmonella species*. Infection varies based on the challenge dose and the animal's immune status, influenced by factors like colostrum intake, prior infections, and exposure to stressors, particularly in elder animals (Adem *et al*., 2022; Kahsay *et al*., 2023).

2.2.2. Environmental and management risk factors

There are two known factors that raise a farm's risk of contracting Salmonella: the presence of other livestock and inappropriate disposal of poultry waste (Jibril *et al*, 2020). Crowding, malnutrition, and other stressful conditions, along with unsanitary surroundings, can worsen mortality and performance declines caused by salmonellosis, particularly in young birds (Mahmud *et al.,* 2015).The presence of chicken dung and other wet organic materials aids in the survival and proliferation of pathogens due to the necessary nutrition and physical protection they offer (Gantois *et al.,* 2009).

Microorganisms in the environment have a longer lifespan when organic material is present, and the quantity of organic matter on surfaces can impact the effectiveness of chemical disinfectants (Hancox *et al*. 2013). Temperature and wetness are most important, as Salmonellas are susceptible to drying and sunlight.

Due to regular immunization programs, proper ventilation, adequate spacing between poultry houses, and no mixing of breeds, the low frequency in intensive farms may be attributed to these factors. In contrast, home agricultural systems may not implement these practices, and historically, incorrect pharmaceuticals in terms of type or dosage were used. Consequently, hens in such systems may remain carriers of diseases despite developing resistance to them (Berhe *et al.,* 2012).

2.2.3 Pathogen risk factors

Salmonella's ability to invade and survive within cells, particularly macrophages, plays a crucial role in its pathogenicity (Marcus *et al*., 2000). The gastrointestinal tract is the main site for the proliferation of these bacteria, with bacterial excretion in feces leading to significant environmental contamination. Once they enter through the intestinal mucosa, cecal tonsils, and Peyer's patches, the organisms are engulfed by macrophages. Subsequently, they spread through the bloodstream and/or lymphatic systems to organs abundant in reticuloendothelial tissues (RES), like the spleen and liver, where they primarily multiply (Abdou *et al*., 2022). Lipid A plays a role in Salmonella's pathogenic properties. One element believed to promote cytokine production is LPS) (Cui *et al.,* 2022).

Salmonella pathogenesis is greatly affected by various virulence markers and determinants like flagella, capsules, plasmids, adhesion systems, and type 3 secretion systems. These mechanisms enable the bacteria to invade, attach to, and bypass the host's intestinal defense mechanisms, such as gastric acid (Chaudhari *et al*., 2023). The virulence of Salmonella is related to their ability to invade host cell and resist both digestion by phagocytes and destruction by complement system (Kemal *et al*., 2015) . The Salmonella virulence strain (specific antigen) benefits from the specific O antigen as it reduces its sensitivity to phagocytosis and enhances its ability to activate the alternative complement pathway (Koo *et al*., 1984). Three toxins namely endotoxin, enterotoxin, and cytotoxic are crucial to Salmonella's pathophysiology. Endotoxins cause fever, enterotoxins cause mucosal injury in cell cultures, and cytotoxins prevent the creation of proteins (Kemal *et al*., 2015).

Salmonella is a genus of facultative intracellular bacteria that dwell in the phagolysosome of macrophages and exhibit resistance to the bactericidal impacts of antibodies. Compared to its relatives, salmonella demonstrates a higher tolerance for diverse environmental conditions. This bacterium can thrive in a pH range of 4–8, temperatures ranging from 8 to 45°C, and with water activities exceeding 0.94. Additionally, Salmonella can propagate in environments with low or absence of oxygen (Eguale *et al*., 2016).

2.2.4 Salmonella's Source

The most common sources of human Salmonella infection are a range of food products, including poultry and other meat products, while outbreaks have also been linked to water (Mishu *et al.,* 1994). There are numerous ways for Salmonella to enter an animal population. These comprise animal feed, the surrounding area, things bought, and other animals that might have access to the farms (White and McDermott, 2009). To prevent environmental contamination and contamination of poultry meat, decontamination procedures such as cleaning and sanitizing the processing facility and machines need to be applied. This is crucial to eradicate Salmonella from poultry carcasses and prevent its spread from carriers to chicken meat (Khan and Rahman, 2022).

2.2.4.1 Sources of infection for animals

Intensively reared food animals, particularly poultry, are a significant contributor to Salmonella infections in various countries. While many animals may not exhibit any symptoms of Salmonella infection, the majority of those infected become carriers without showing clinical signs (Rahman *et al*. 2018). The most prevalent animals that serve as reservoirs include pheasants, quails, sparrows, parrots, chickens, turkeys, and guinea pigs (Quinn *et al*., 1994). During the production cycle, poultry can become infected with Salmonella through various routes, including contact with carrier animals like rodents, cats, and insects. Contaminated poultry feed, litter, water, and aerosol transmission also contribute to the transmission of Salmonella (Syamily *et al,* 2023).

2.2.4.2 Sources of infection for human beings

Salmonella is mainly found in the intestines of humans who are sick, recovering or not showing any visible symptoms of the illness. These individuals shed the bacteria which can contaminate water sources, and eventually spread to poultry and other food products that are exposed to the contaminated water (Khan and Rahman, 2022). The main source of infection for humans is poultry products (meat and eggs), which are frequently derived from healthy animals (Gast *et al*., 2024). Many pathways can lead to human transmission. Important pathways for pathogens to contaminate food products include eating contaminated food products (milk, eggs, and meats), coming into direct contact with animals and their environments, and cross-contaminating food products by coming into direct contact with contaminated surfaces like stainless steel, hanging materials, knives, and buckets used to collect milk (Pal *et al*., 2015).

Public health concerns extend to foodborne illnesses in both developed and developing countries such as Ethiopia. Food contamination risks exist during various stages of the food supply chain like production, processing, distribution, and preparation. Maintaining high standards of personal hygiene is essential in dairy farms and food processing facilities (Eguale *et al.,* 2016).

2.2.4.3 Carrier state

Among the animals raised for food, poultry is the main source of various NTS serotypes. Some of the NTS serotypes that are epidemiologically important include *S. typhimurium, S. enteritidis, S. heidelberg*, and *S. newport*. *S. enteritidis* is the most prevalent serovar associated with egg-borne infection in North America and Europe, while *S. typhimurium* was the primary serovar associated with external egg contamination (Dar *et al*., 2017; Chousalkar *et al*., 2018).

2. 3. Clinical picture

2.3. 1. In poultry

Symptoms, which included diarrhea, dehydration, decreased body weight gain, lameness, and significant mortalities, were primarily seen in one-week-old broiler chicks. Hepatitis,

hepatomegaly with necrotic foci, arthritis, typhlitis, omphalitis, myocarditis, and pneumonia were the predominant postmortem pathologies. However, the symptoms were less severe in older birds at the 6^{th} week of age (Ahmed, 2022). The severity of *Salmonella* infection varies according to many factors, including host age, host immunity, the presence of coinfections, environmental stress, managerial characteristics, and infective dose. Older birds, for instance, tend to be less susceptible to Salmonellosis even with concentrations of 10^6 CFU/mL of pathogenic *Salmonella species (*Syamily *et al., 2023).* Clinical indicators such as decreased feed intake, drooping, fluffed feathers, huddled chicks with diarrhea, dehydration, and mortality were noted. At necropsy, the livers were always found to be infected with *Salmonella species*. and to be swollen, squishy in substance, congested, and discolored with bronze (Hyeon *et al*., 2010).

2.3.2. In humans

Humans can contract salmonella infections in a variety of ways, from typhoidal fever with potentially fatal intestinal perforations to self-limited gastroenteritis typically caused by NTS (OIE, 2019). Salmonella infections can present in various clinical forms including enteric fever, gastroenteritis, bacteraemia, extra-intestinal localized infection, and a chronic enteric or urinary carrier state. The major risk factors for nontyphoidal salmonellosis and bacteremia include extremes of age, alteration of the endogenous bowel flora of the intestine, diabetes mellitus, malignancy, autoimmune disorders, and blockage of the reticuloendothelial system, HIV infection, and therapeutic immunodeficiency. These risk factors can make individuals more susceptible to Salmonella infections and may increase the severity of the illness. Vigilance and prevention are key in avoiding Salmonella infections. It is vital to practice good hygiene, including frequent hand washing, and to ensure proper food handling and storage techniques. It is also important to seek medical attention if you experience symptoms of a Salmonella infection (Dhanoa and Fatt, 2009).

Typhoid and paratyphoid strains, well-adapted for invasion and survival in human hosts, are associated with enteric (typhoid) fever, a dangerous illness. Gastroenteritis is a common side effect of nontyphoid salmonella's infection, typically appearing 6-72 hours after exposure to the invasive bacterium (Urfer *et al.*, 2000). Clinically, enteric fever,

septicemia, or gastroenteritis may present as symptoms of salmonellosis. Enteric fevers are caused by *S. enterica serovars typhi* and *paratyphi*, which are infections specific to humans. The severity of infection can vary based on an individual's immune system, resistance, and the virulence of the Salmonella isolate (Andino and Hanning, 2015).

Within five days following the commencement of symptoms, the characteristic non-bloody diarrheal stools and abdominal pain usually go away, indicating that the clinical condition is typically self-limiting. Nontyphoid strain infections in humans can also progress into systemic infections and cause a number of chronic illnesses, including ankylosing spondylitis and aseptic reactive arthritis. When invasive salmonellae are present in a human host, preexisting physiological, anatomical, and immunological problems may make the sickness more severe and prolonged by impairing the host's defense mechanisms (Lai *et al.*, 2005). Salmonella infections range from gastrointestinal infections characterized by inflammation of intestinal epithelia, diarrhea, and vomiting to typhoid fever, a potentially fatal infection whose severity is determined by the host immune status and the pathogenicity of the bacterium consumption of *Salmonella (*van Asten and van Dijk, 2005*).* Contaminated foodstuffs result in gastroenteritis, bacteremia and extra intestinal focal infection in infants like meningitis and may also cause severe illness among the elderly and immune-compromised patients (Saravanan *et al.*, 2015).

2.4. Health Impact of Salmonellosis

Salmonella typically causes diarrhea in people (Tariq1 *et al.*, 2022). *Salmonella species* can lead to illness in humans and animals through various serovars. Presently, there are approximately 2,579 recognized *Salmonella serovars* (WHO.2007). Among these, *S. typhimurium* is the most commonly isolated serovar in humans, followed by *S. enteritidis;* conversely, *S. typhimurium* is the most commonly isolated serovar of nonhuman origin, followed by *S. newport* (Silva *et al*., 2014). . Of all the *Salmonella enterica serovar*s, *Salmonella typhimurium* is most frequently linked to intestinal illnesses in both humans and animals. Humans, cattle, pigs, sheep, horses, rodents, and birds are among the many hosts that this serovar can infect. According to Van Parys *et al*. (2011), the three most common serotypes of Salmonella that are typically recovered from humans each year are *Salmonella enteritidis, Salmonella typhimurium*, and *Salmonella*

heilberg. Since poultry are known to be the main source of non-host adapted salmonellosis in humans, *Salmonella typhimurium* and *Salmonella enteritidis* not only pose a serious risk to public health but also play a major role in severe outbreaks of avian salmonellosis and cause significant financial losses to the poultry industry. *Salmonella enterica* infection is one of the most significant foodborne zoonoses in the world, causing gastroenteritis in humans. A significant percentage of infections in humans are caused by multidrug-resistant *Salmonella typhimurium* definitive phage type (DT) 104 strains, which are largely zoonotic in origin (Obe *et al*., 2018). Salmonella serotypes are harmful to humans, with common symptoms including fever, fatigue, muscle pain, diarrhea, nausea, and vomiting. Factors such as inoculation dose, pathogenicity mechanisms, virulence factors, age, and host immune response can influence the symptoms experienced (Andino and Hanning, 2015).

2. 5. Economic Impact of Salmonellosis

There is a significant burden of foodborne illnesses: 33 million years of healthy living are wasted and nearly one in ten individuals become ill annually. According to CDC estimates, each year in the US, Salmonella causes over 1.2 million infections, 23,000 hospitalizations, and 450 fatalities. Approximately one million of these diseases are caused by food (CDC, 2018). The worldwide spread of salmonella, its substantial economic impact, and the difficulties in controlling the disease make it a major poultry disease affecting birds (Kabir, 2010). A Salmonella control program has been implemented for a number of years in the nation; the anticipated yearly expenditure of this program is $14.1 million (Mahmud *et al*., 2015). Salmonellosis costs billions annually in the US, hundreds of thousands to millions in Canada, and millions in the UK. A study on five North American Salmonella outbreaks from tainted food showed costs from 36,400 *to* 36,400 to 62 million. Research on preventing Salmonella infection's costs and benefits is limited, but investing £1 in outbreak research and containment could save $5 (Wrap, 1994).

This disease results in high mortality rates, which can approach 100%, a drop in egg and chick production, the condemnation of the afflicted carcass, and expensive medical expenses for both humans and animals. These factors together account for significant

economic losses. Estimates are produced as part of a multidisciplinary job that includes direct health expenditures including hospitalization, doctor consultations, laboratory tests, and lost labor related to a salmonellosis case (Berhe *et al.* 2012). Salmonellosis causes severe economic damage to chicken production by reducing production, 100% morbidity, and 20% mortality in affected flocks (Hald *et al*., 2012).

2. 6. Diagnosis of Salmonella

Diagnosis depends on detecting Salmonella from feedstuffs, food items, environmental samples, rectal swabs, and aseptically recovered skin or tissues after necropsy, as well as food products. Serological tests may identify past or current animal infections by specifics (Badr *et al*., 2021). Various techniques can help in identifying organisms, such as using selective agars to differentiate Salmonellae from other enterobacteria, employing pre-enrichment to revive sub-lethally damaged Salmonellae, and utilizing enrichment media with inhibitory substances to impede competing organisms. An isolated strain can be accurately confirmed through a range of biochemical, serological, and molecular tests on the pure culture. Typing sera specific for antigens - termed somatic (O), flagellar (H), and virulence (Vi) - can be employed to identify the organism. Serovar determination can be done using antigenic formulas within the Kaufman-White scheme (Sodagari *et al*., 2020).

2.6.*1 Clinical Diagnosis*

The majority of the tentative diagnosis is based on post-mortem, mortality, flock history, and clinical symptoms. Huddling beneath a heat source, anorexia, depression, and white faeces pasted around their vents are the most prevalent clinical symptoms of pullorum illness in infected birds. When the birds are between two and three weeks old, the death rate is high-up to 100%. Lesions that are characteristic include localized necrosis of the liver and spleen, white nodes throughout the lungs, and unabsorbed yolk (Berhanu and Fulasa, 2020).

2.6.2 Laboratory Diagnosis

Salmonella testing on chicken lowers the risk of outbreaks and safeguards public health by identifying tainted goods and keeping them from reaching consumers (Munster *et al*., 2023). Although *S. gallinarum* and *S. pullorum* must be isolated and identified to conclusively diagnose Fowl Typhoid and Pullorum Disease. Clinical signs, flock history, mortality, and lesions can all help make a preliminary diagnosis. An ELISA specific to this purpose can be used in a lab or as a pen-side test that can be visually interpreted with appropriate controls. To differentiate from positive reactions due to *S. enteritidis* infection, the ELISA has been employed for both serovars using a combination of group D lipopolysaccharides (LPS) and flagella antigen (Barrow and Neto, 2011).

Many factors like government regulations, examination goals, cost-benefit analysis, equipment facilities, reagent availability, and staff experience impact and at times restrict the selection of lab methods. Presently, legislation predominantly mandates the isolation of the organism. Nevertheless, voluntary control programs might employ serology or alternative methods to identify infected flocks. Clinical signs and lesions hold minimal diagnostic value, given instances where infection lacks clinical disease or lesions (Hafez, 2001).

2.6.2.1 Pre-enrichment in non-selective liquid medium

The ability to recover Salmonella from food, water, environmental samples, or animal sources can be significantly impacted by enrichment methods. The technique chosen will depend on whether non-selective nutritional broths, like trypticase soy broth or buffered peptone water (BPW), are required to revive damaged cells from samples that have been heat-stressed, dehydrated, or in other less-than-ideal conditions (Torrence and Isaacson, 2003).

2.6. 2.2 Enrichment in selective liquid media

Enrichment in selective liquid medium may follow an initial enrichment in non-selective liquid media or it may be used as an initial step in specimens containing high numbers of competing bacteria such as faeces or ground meats to prevent overgrowth by coliforms

that can readily outcompete the Salmonella (Torrence and Isaacson, 2003). Enrichment is done by the transfer of 0.1 ml of the culture obtained in nonselective preenrichment medium to a tube containing 10 ml of a Rappaport-Vassiliadis magnesium chloride malachite green medium (RV medium) (ISO, 2022). And transfer of another 10 ml to a flask containing 100 ml of selenite cystine medium or 1 ml mixture to 10 ml tetrathionate (TT) broth. The inoculated RV medium is incubated at 42^{o}C for 18 to 24 h or 24 ± 2 h at 42 ± 0.2°C. And selenite cystine medium at 35^{o}C to 37^{0}C 18h to 24h. The TT broth is incubated at 24 ± 2 h at 43 ± 0.2°C.

2.6.3.3 Plating Out and Identification

Cultures obtained from the selective enrichment are streaked onto two selective media: − Xylose lysine deoxycholate agar (XLD agar) and, for testing as part of export certification, any other solid selective medium that is complementary to XLD and able to detect H_2S negative serovars of Salmonella e.g. Brilliant Green Agar (BGA). XLD agar is incubated at 37 ± 1^{o}C and examined after 24 h ± 3 h. The second agar is incubated according to the manufacturer's recommendations. It does not require the use of duplicate 90 to 100 mm Petri dishes or a single 140 mm Petri dishes. Single 90 to 100 mm Petri dishes can be used. Confirmation can be directly off the selective agar if well isolated colonies are available (ISO, 2022).

2.6.3.4 Confirmation

Presumptive Salmonella colonies are subculture and confirmed by means of appropriate Biochemical and serological tests. For confirmation typical or suspect colonies are streaked onto the surface of pre-dried tryptic soy agar plates, in a manner, which will allow well-isolated colonies to develop. Pure cultures are used for biochemical and serological confirmation (ISO, 6579-1:2017/Amd.1:2020(E), 2020).

Biochemical Confirmation*:*

Salmonellae are a type of bacteria that are chemoorganotrophic, meaning they obtain energy by metabolizing organic compounds. They are able to use both respiratory and fermentative pathways to break down nutrients. In particular, they are known to consume

D-glucose and other carbohydrates and produce acid and gas in the process. Salmonellae are negative for the enzyme oxidase, indicating their inability to catalyze oxidase reactions, but they are positive for catalase, which helps them to break down hydrogen peroxide. These bacteria can grow using citrate as the sole carbon source, and they generally produce hydrogen sulphide. They have the ability to decarboxylate lysine and ornithine, but they do not hydrolyze urea (Quinn *et al*., 1994).

Molecular Techniques*:*

A variety of rapid identification and sensitivity methods have been developed for isolates from clinical samples; these include molecular techniques such as Real-time Polymerase Chain reaction (PCR), Pulsed Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Multiple-Locus Variable-Number TandemRepeat Analysis (MVLA), SNP assays, Whole Genome Sequencing (WGS) and Matrix Assisted Laser Desorption Ionisation Time-of-Flight (MALDI-TOF) Mass Spectrometry. All of these approaches enable subtyping of unrelated strains, but do so with different accuracy, discriminatory power, and reproducibility (Public Health England (PHE), 2024).

2.7. Biosecurity in the Control of Salmonella in Poultry

2.7.1. What is biosecurity?

The World Organization for Animal Health (OIE, 2010) defines biosecurity in the Terrestrial Animal Health code as "a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal diseases, infections or infestations to, from and within an animal population.” The term "biosecurity" in the context of poultry refers to a collection of procedures and controls used to restrict, manage, or stop the entry and spread of infectious diseases within the buildings and grounds of poultry (Anne, 2012; Eltholth *et al.*, 2016; Scott *et al*., 2018). A biosecurity program uses specific measures like thorough car washing, footbath usage, and disinfection of farm equipment, as well as physical barriers such as fences and wire mesh to prevent the introduction or minimize the transmission of disease-causing organisms (Aiyedun, 2018).

Three phases of biosecurity can be implemented: isolation, which focuses on shielding hens from infectious agents; traffic control, which restricts traffic flow; and sanitation control, which involves sanitizing and restricting the movement of equipment (Terfa *et al*., 2015) and operational frameworks of biosecurity measures include house design and construction along with management practices that prevent infectious diseases from infecting the flock (Halvorson, 2011 and Maduka *et al*., 2016). Biosecurity is a way to prevent diseases from entering and spreading within a country, area, or farm. In poultry farms, it involves practices to keep birds away from sources of disease such as people, animals, equipment, and feed. The goal is to ensure the birds' health and productivity (Jay, 2008). The Food and Agriculture Organization of the United Nations (FAO) defines biosecurity as "Implementation of measures that reduce the risk of the introduction and spread of disease agents; requires the adoption of a set of attitudes and behaviors by people to reduce risk in all activities involving domestic, captive/exotic, and wild animals and their products" (Huber *et al.*, 2022).

2.7.2 Explanation of Poultry Farm Biosecurity

Farm biosecurity includes "bio exclusion," which refers to steps taken to keep a pathogen from entering a herd or flock, and "bio containment," which deals with what happens after introduction, such as the potential for a pathogen to spread among animal groups on a farm or, more generally, in terms of releases from the farm (Dargatz *et al.,* 2002). The mix of individual bio-exclusion procedures and bio-containment methods determines how diseases spread between farms. Biosecurity must be included into all aspects of farm operations (Manuja *et al*., 2014).

In poultry farms, biosecurity is the first line of defense against the introduction and spread of viruses that could have negative effects on food safety, animal health, and the bottom line (Tilli *et al.,* 2022). Biosecurity at a poultry farm includes all measures taken to minimize the risk of introduction and spread of disease agents and thus, includes all actions for keeping poultry and the farm healthy. By taking these biosecurity measures and performing efficient management, on-farm animals are protected against both endemic and epidemic diseases (Dewulf and Van Immerseel, 2018). The goal of external biosecurity is to stop diseases from entering or leaving the farm by concentrating on the

points of interaction between the farm and the outside world. This is true for endemic illnesses, which are prevalent in a nation but do not affect every farm, as well as exotic diseases, which are uncommon in a nation (Ribbens *et al.,* 2008). Internal biosecurity encompasses all actions required to prevent the spread of infections on a farm (Joint, 2016, Laanen *et al*., 2013).

According to the United States Department of Agriculture, Animal and Plant Health Inspection Service (2019), biosecurity measures at farm level include installing units in low-landhold areas, establishing sanitary barriers, controlling vermin, cleaning poultry houses and equipment, collecting hatching eggs, and distributing newly hatched poultry in clean boxes and papers. These measures also involve monitoring staff and visitors, ensuring food safety, and ensuring proper handling of birds and crates after each use. The study conducted in Egypt's Sharkia Governorate revealed that many farms lacked proper biosecurity protocols, which led to increased Salmonella isolation rates. It underlined the necessity of better biosecurity measures to reduce the dangers of Salmonella infections (Attia, 2023).

2.7.3. Quantification of the Biosecurity Level

The Biocheck.UGent system is a risk-based scoring system to quantify the on-farm biosecurity. It tackles biosecurity generally and concentrates on the factors that are common for the spread of numerous infectious disease types rather than beginning with a particular illness. For internal and external biosecurity, the surveys are broken down into multiple subcategories, each with two to nineteen questions. The majority of questions include two or three possible answers. A score ranging from zero (when this measure is not implemented at all or the least optimal answer is supplied) to one (when the measure is completely applied) is assigned to each question's response. Question scores are multiplied by a weight factor based on the significance of a certain biosecurity metric (Gelaude *et al.,* 2014).

The Biocheck.UGent™ scoring system was developed to provide a risk-based weighted assessment of biosecurity measures across different poultry types. This involved creating five distinct questionnaires tailored for breeder, turkey, duck, free-range layer, and free-range broiler production (Amalraj *et al.,* 2024). Biocheck is a risk-based, impartial

system that was created by Ghent University in Belgium to assess the level of on-farm biosecurity. Several European nations have employed the Biocheck. UGent scoring tool to evaluate the biosecurity of livestock farms.

2.7.3.1. External Biosecurity

As Ribbens *et al.* (2008) state, external biosecurity focuses on the locations where the farm interacts with the outside world and seeks to stop diseases from entering or leaving the farm. This encompasses both exotic diseases, which are uncommon in a nation, and endemic diseases, which are prevalent in a nation but not present at every farm. The first step in putting the required changes into place to make layer farms more resilient to the threat of disease incursion may be determining the true level of biosecurity in these farms (Jarkko and Lehtonen, 2009).

Purchase and Introduction of New Animals:

The fundamental principles of external biosecurity are isolating new animals, following quarantine protocols, conducting disease tests, taking preventive actions, and maintaining hygiene (Barrington *et al*., 2006). Buying from marketplaces or dealers poses a serious danger to biosecurity. When animals are introduced or reintroduced onto farm property, proper quarantine and testing are part of the animal health procedures. Disease-causing pathogens may be introduced if animals are purchased or reintroduced after attending fairs, exhibitions, etc. (Manuja *et al.*, 2014).

Disposal of Manure and Disposal of Carcasses:

Biosecurity measures include manure management plan to address collection, storage, handling and disposal. It should be outside of the production area. Biological composting and anaerobic storage are required before spreading manure in the fields. It should also be ensured that neighboring producers do not spread manure adjacent to production areas or water sources. Contaminated beddings, animal products, manure, feed and fodder residues also need to be disposed away from the farm area (Manuja *et al.,* 2014). The possibility of Salmonella reintroduction through fomites, improper waste disposal is believed to raise the risk of Salmonella infection in chicken farms. According to specific

research, a farm's risk of testing positive for Salmonella is increased thrice when trash is disposed of improperly (Jibril *et al.*, 2020).

Feeding and Watering Management*:*

The introduction and spread of infections can occur through contact with contaminated bedding, polluted water, or contaminated feed. In humid and tropical settings, feed frequently becomes contaminated with fungus and associated poisons. It is crucial to make sure the feed is free of contaminants. Periodically and prior to usage, the quality of the feed and bedding materials should be examined. It is crucial to do routine testing of water, soil, feed, and fodder when putting a biosecurity plan into action at the farm level. Regular cleaning is necessary because water supplies can get contaminated with urine or feces, which can expose animals to germs that cause sickness. *Salmonella, Escherichia coli O157,* and *Campylobacter* can live for up to three months in slurry and contaminated water, making them biohazards (Nicholson *et al*., 2005). Contaminated feed and water can serve as pathways for introducing diseases into poultry flocks, highlighting the need for comprehensive biosecurity measures throughout the production process (Robertson, 2020).

Farm site in relation to infrastructure and biological vector control*:*

If the farm is close to other farms, slaughterhouses, livestock markets, waste disposal facilities, hatcheries, and corpse centers, the risk of contracting diseases increases significantly. The risk is increased by the location's proximity to waterways and animal transportation routes. In order to reduce the risk of disease entry and dissemination, barns, buildings, ventilation inlets and outlets, unloading and loading places, and treatment and isolation or quarantine locations should be oriented accordingly (Manuja *et al.*, 2014). The role of carrier vectors in the transmission of Salmonella and other pathogens is well-established and widely discussed in the field of veterinary and public health. Various animals, including rodents, birds, insects, feral animals, dogs, and cats, can mechanically transmit these pathogens. Among these, rodents are particularly significant due to their ability to harbor and spread infections (Andres and Davies, 2015).

2.7.3.2. Internal Biosecurity

The development and spread of diseases both within and between populations are accompanied by a variety of risk factors. It is rational and practical to group these risk variables according to how they affect host animals, the environment, or infectious pathogens. Before implementing specific bio-containment procedures intended to lessen disease, it is essential to understand both the existence and the significance of particular risk factors (Barrington, 2014). To reduce the possibility of infectious organisms spreading to other flocks, facilities, and people, staff should manage flocks. The management of an infected flock separately, last in line, and the use of specialized staff, clothing, and equipment are all pertinent procedures (OIE, 2022).

As the study revealed by (Tranquility *et al*., 2020) values for cleaning and disinfection, materials and measures between compartments, and internal biosecurity subcategories are higher than the overall values. The variables include disease management, cleaning and disinfection, material, and measures between compartments (Gelaude *et al*., 2014).

Cleaning, disinfection and sanitation measures*:*

Disinfection is the process of physically and chemically eliminating pathogenic bacteria. In order to prevent the spread of infectious materials and to limit the entrance of disease-causing agents to the flock, hygiene entails the construction of physical barriers (Chima *et al*., 2012). Cleaning decreased the bacterial load; the initial disinfection stage, which used either formaldehyde fogging or peracetic acid, further decreased but did not completely eradicate Salmonella, while the air supply systems remained positive. Although Salmonella did remain in trace amounts in the external environment, the addition of a second disinfection stage utilizing sodium hypochlorite and external lime application totally eradicated the disease on one farm and inside the second's internal buildings (Alarcon-Lopez *et al.,* 2021).

Cleaning and disinfection of production areas, quarantine sheds and equipment’s after each production cycle are helpful. Shared and reusable equipment’s between animals need to be cleaned and disinfected before and after use. Animal feeders, water channels and feeding areas should be cleaned regularly. Cleaning and disinfection of feed delivery equipment’s that are in contact with mortalities, manure or feed are some of the measures

relating to biosecurity considerations on a farm. Disinfection of liquid effluents from contaminated areas should also be performed before disposal. Certified and internationally accepted disinfectants must be used at farm premises (Manuja *et al.*, 2014). Effective removal of pathogens or niches favorable for their survival is critical to prevent disease spread to neighbors and maintain good hygiene (Tadesse *et al.*, 2024).

Identification and treatment of sick animals:

In order to lessen the impact on an operation and aid in the prevention of disease spread, the capacity to respond promptly and effectively to a disease situation is essential. An early diagnosis of a disease that warrants worry can lessen its effects and make containment easier. Disease can be easily spread by sick animals through direct or indirect contact. These animals need to be kept apart from healthy animals while being examined. Positive reactors and reservoirs for specific diseases should be removed from the herd since they can shed the infection and infect other animals. To determine the health status of the herd, routine disease testing and screening of animals should be done. The likelihood of a disease spreading to a farm can be decreased by taking preventative precautions. The effectiveness of treatment can be increased with proper pharmaceutical use. Schedules for vaccinations and deworming should be created and strictly adhered to. It is important to keep accurate records of treatments and vaccines (Manuja *et al.*, 2014).

2.7.4. Correlation between biosecurity and Salmonella presence

Farms with poor biosecurity scores exhibited a higher incidence of Salmonella compared to those with good biosecurity practices. The finding underscores the importance of implementing robust biosecurity measures to mitigate production losses and reduce zoonotic risks associated with bacterial infections like salmonellosis (Muluneh, 2021). Implementing robust biosecurity measures in poultry farming is essential to reduce the prevalence of Salmonella and enhance food safety, as routine monitoring and testing of farms are crucial for ensuring compliance with biosecurity standards, thereby protecting both animal and human health from zoonotic diseases linked to Salmonella (Attia, 2023).

2.7.5 Biosecurity practices in Ethiopia

In Ethiopia, as in other Sub-Saharan African countries, biosecurity measures are often inadequately implemented (Alemu, 2008). Reliable data on the health practices of commercial poultry farms in central Ethiopia remain limited, highlighting the need for comprehensive studies to evaluate biosecurity practices and develop effective preventive strategies tailored to farm sizes (Asfaw *et al*., 2019).

The lack of proper biosecurity measures poses significant risks to farm productivity and public health, particularly through the potential spread of zoonotic diseases (Tsegaye *et al*., 2023). Studies in Bishoftu Town revealed that many commercial chicken farms lack proper biosecurity protocols, with only a few farm owners receiving training. Issues such as poor site selection, inadequate feed provision, and careless sourcing of day-old chicks contribute to these shortcomings (Abdurehman *et al*., 2023). Seasonal disease outbreaks and insufficient biosecurity measures further challenge Ethiopia's poultry sector. For instance, research by Tadesse *et al.* (2024) found that only 23.5% of central Ethiopian poultry farms achieved good biosecurity scores, while 76.5% had poor practices. Similarly, small- and medium-scale farms in Addis Ababa demonstrated significant gaps in biosecurity, which were linked to higher incidences of Salmonella infections. Farms with inadequate protocols had a greater prevalence of Salmonella compared to those with effective measures (Ayele, 2021). Insufficient biosecurity practices have also been observed in small-scale commercial poultry farms in Debre Markos, where many lacked isolation facilities for sick birds, visitor logs, or footbaths at entrances, increasing the risk of disease transmission (Yitbarek *et al*., 2016). Moreover, breeding and multiplication facilities for poultry in Ethiopia are major sources of infections, including drug-resistant Salmonella strains. These infections often stem from environmental factors such as contaminated bedding, human contact, and infected poultry (Abdi *et al*., 2017). The spread of Salmonella is further exacerbated by inadequate pest control measures and poor management practices, such as insufficient record-keeping and delayed responses to health issues. Rodent infestations and lack of pest treatments also contribute to Salmonella transmission (Abdelgadir and Ismail, 2017).

Improving biosecurity practices is essential to mitigate these risks. Routine flock testing, enhanced farm surveillance, stricter regulations on antimicrobial use, and policy support

are critical for controlling Salmonella in poultry farming (Waktole *et al*., 2024). Studies in Jimma, Ethiopia, confirm the strong correlation between Salmonella infections and inadequate biosecurity measures, with poor management being a key contributor to infectious diseases (Kabeta *et al*., 2024).

To reduce Salmonella infections, farms must adopt comprehensive management plans, improve biosecurity protocols, and promote training and awareness programs. Strengthening biosecurity measures both internal and external combined with better knowledge of mycotoxin control can significantly reduce disease risks (Terio *et al*., 2023). The low biosecurity levels in central Ethiopian poultry farms underline the need for collaborative efforts from all stakeholders to implement effective practices, safeguard public health, and enhance the productivity of the poultry sector (Birhanu et al., 2015; Waktole *et al*., 2023).

3. MATERIALS AND METHODS

3.1. Description of the Study Areas

Mekelle, situated 780 Kilometers north of Addis Ababa, is both a special zone and the capital of the Tigray Region in Ethiopia, boasting an elevation of 2,254 m.a.s.l above sea level. Functioning as the economic, cultural, and political nucleus of northern Ethiopia, Mekelle is administratively designated as a Special Zone (Aberra, 2007). The town's population, according to the 2023 population projection by the Central Statistical Agency of Ethiopia (CSA), stands at 480,198, comprising 243,635 men and 236,563women (CSA, 2023). Positioned in the northern region of the Ethiopian Rift Valley on a Jurassic limestone plateau, Mekelle experiences a semi-arid climate, with an average annual rainfall of 714 millimeters (28.1 in) at the airport (Britannia, 2020). Mekelle is further subdivided into seven local administrations-Hawelti, Adi-Haki, Kedamay Weyane, Hadnet, Ayder, Semien, and Quiha. These local administrations consist of kebeles or ketenas. Each sub-city operates as a borough with its own administrator, though it falls under the governance of the Mekelle Special Zone Government. These authorities are responsible for managing health, education, utilities, and overseeing socioeconomic development (Habtu, 2011).

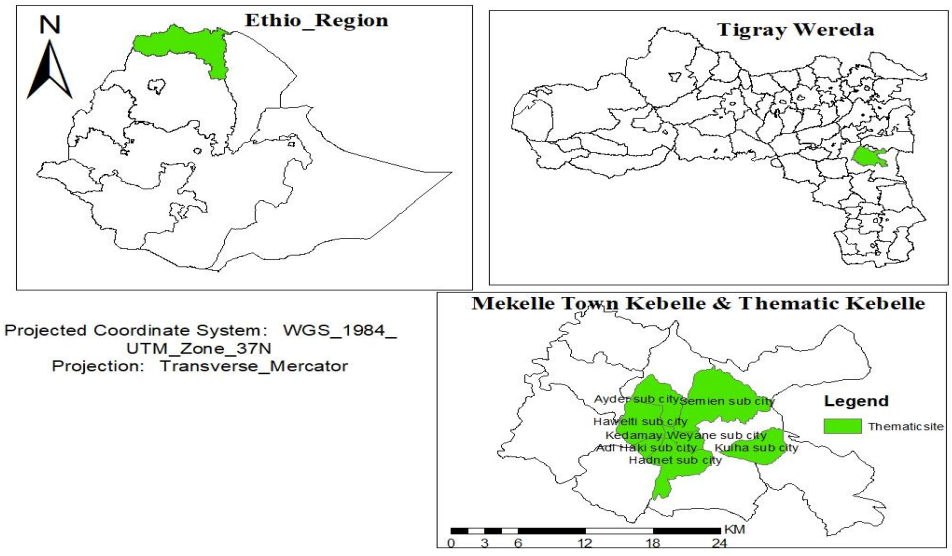


Figure 1. Map of study area

3.2 Study Population and animals

The target population includes small and medium-scale commercial laying poultry farms located in selected sub-cities of Mekelle City. These farms have been established by a mix of private enterprises, micro and small-scale businesses, as well as cooperatives. The study animal focused on commercial chicken which predominantly raise exotic chicken breeds, apparently healthy, specifically Bovans brown and saso with different age groups flock sizes and maintained under an intensive management system. These farms are categorized by size: small-scale (fewer than 1,000 birds), medium-scale (1,000 to 10,000 birds), and large-scale (more than 10,000 birds) (Alemayehu and Tesfaye 2020).

The exotic breeds raised in these farms are taken from Ethiochicken and are known for their higher productivity compared to indigenous chicken breeds, particularly in small and medium-sized commercial poultry operations. The zone agricultural coordinator provided statistics that estimated there were approximately 300 medium- and small-scale intensive poultry producers (farms) with a total population of 122,656 chickens. It was challenging to determine the precise number of operational poultry farms by specific classification despite efforts to create a comprehensive and up-to-date list because of the high turnover rate of poultry farms brought on by elements like market fluctuations, ownership changes, war crises, and fluctuating levels of farm activity.

3.3. Sample size determination

For isolation and identification of Salmonella, the minimum required sample size for this study was calculated using a formula described by Thrusfield (2007), based on the sample-level prevalence of Salmonella reported in Mekelle Ethiopia by Ashawani et al. (2014), which was 19.71%. The sample size calculation assumed a 95% confidence level and 5% absolute precision. The formula used for calculating sample size in prevalence studies was: $n = (Z^2 \times P \times (1 - P))/d^2$; Where: n = required sample size, Z = value corresponding to the desired confidence level (1.96 for 95% confidence), P = expected prevalence (19.71% or 0.1971), and d = desired absolute precision (5% or 0.05). Based on the above formula the minimum sample size was calculated total of 243 laying hens were sampled. To increase the precision of the estimate, the sample size was increased to

a total of 250 laying hens sample. These were from 28 different poultry farms. From each farm, a representative person was interviewed for biosecurity practices checklists. Farms were purposively selected due to their availability, owner desire, and the substantial potential for poultry production, as well as the availability of small and medium-sized commercial layer hen farms that were currently producing eggs.

3.4. Study Design and Sampling Techniques

A cross-sectional study was conducted from December 2023 to December 2024 in Mekelle city, Tigray Region, to evaluate the prevalence and molecular detection of Salmonella isolates in small- and medium-scale commercial laying hens in Mekelle city, as well as to assess biosecurity measures in selected small and medium scale commercial laying hens of the study farms. When birds were sampled, they must not exhibit any clinical symptoms of illness. To choose laying hens from the selected farms, simple random selection was used. The number of hens selected for sampling was proportional to the size of each farm. This approach minimized sampling bias and ensured a fair representation of different flock sizes. The total number of samples collected was determined by taking 1% to 3% of the total flock size, with minor modifications allowing for a range of 3% to 5% based on the specific flock size settled by (Dagnew et al., 2020).

3.5. Sample Collection and Transportation

The samples for isolation and characterization of Salmonella were taken from cloaca swab of poultry as indicated in https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7424249/. Swab samples have been taken following the procedure outlined in ISO 6579-1:2017/Amd.1:2020(E) by collecting cloacal swabs using sterile cotton swabs that were moistened in 10 ml of sterile buffered peptone water (BPW). The swabs were gently rotated and suspended in 9 ml of BPW before the wooden shaft was broken off and the cotton swab was left inside the sampling bottle. After that the collected samples were transported and stored at 4 °C and were either immediately processed upon arrival at the laboratory or the day after. Samples were transported to the Laboratory of Microbiology, CVS, and Tigray Regional Veterinary laboratory in an icebox. The samples were then incubated at 37 degrees Celsius for 24 hours.

For the study on Biosecurity measures, the toolkit from the Gent University was translated into Tigrigna language; formatted into digital platforms (google form; Kobo Collect) that can be accessed on android phone. Data entry was made from a willing layer growers and producers.

3.6. Bacteriological Isolation of Salmonella

Salmonella was isolated and identified according to standardized protocols described by the International Organization for Standardization for Salmonella detection in food and animal feedstuffs ISO 6579 ISO 6579-1:2017/Amd.1:2020(E), and Aziza et al. (2018). The bacteriological examination was conducted following the horizontal method for the detection, enumeration, and serotyping of Salmonella. To isolate Salmonella, the standard three-stage process was utilized, which involved pre-enrichment, selective enrichment, and selective plating (Figure 2).

In the primary enrichment step, the collected samples were diluted in buffered peptone water (1:10) and incubated aerobically at 37 °C for 18 hours. Subsequently, 100 microliters of the pre-enriched media were transferred to a tube containing 10 ml of Rappaport Vassiliadis broth and incubated at 41.5 °C for 24 h. During the secondary enrichment step, the Rappaport-Vassiliadis with soya (RVS) was adjusted to room temperature, and the mix incubated in the primary enrichment sample was manually massaged for at least 10 s. A 0.1 ml aliquot was then transferred and added to 10 ml of Rappaport-Vassiliadis with soya (RVS). The tubes were vortexed and incubated at 41.5° for 24 h. After that, the enriched cloaca swab of poultry samples was plated onto Selective Agar. A selective medium, Xylose lysine deoxycholate (XLD) agar (HIMEDIA M031, India), was used for isolating Salmonella and was adjusted to room temperature as per the manufacturer's instructions. The secondary enrichment tubes were vortexed before plating on XLD agar. After adjusting XLD, the samples were streaked from secondary enrichment tubes using a 10 µl loop and incubated at 35 °C for 24 h. Following the recommended incubation time, the selective-differential agar plates were examined for the presence of colonies meeting the description for suspected Salmonella colonies. Typical *Salmonella species*. Colonies appeared as pink colonies with or without black centers on XLD agar. Three to five typical colonies of Salmonella were picked and

streaked onto Trypton soya agar and incubated at 37 °C for 18–24 h for further biochemical identification.

3.6.1. Pre-Enrichment in Non-Selective Broth Medium

According to the ISO 6579-1:2017/Amd.1:2020(E) standard, all samples were pre-enriched separately with an appropriate amount of BPW (CONDA) (1:9), and they were incubated for 18-24 hours at 37 .5°C.

3.6.2. Enrichment in Selective Broth Media

Tetrathionate broth base (Titan Biotech Ltd.) and Rappaport Vassiliadis Salmonella enrichment broth (Himedia MH1491) were both used for selective enrichment of all samples. A portion (1 ml) of the pre-enriched culture was aseptically transferred to 10 ml of tetrathionate broth base containing test tube, and another 0.1-ml pre-enriched culture was aseptically transferred to test tubes containing 10 ml of Rappaport Vassiliadis Salmonella enrichment broth and incubated at 37 ± 1°C and 41.5 ± 0.5°C for 24 h, respectively (ISO 6579-1:2017/Amd.1:2020(E), 2020). From the pre-enriched sample, 0.1 mL was transferred into a tube containing 10 mL of RV broth and incubated at 42 °C for 24 h. Another 1 mL was transferred into a tube containing 10 mL of MKTT broth and incubated at 37 °C for 24 h.

3.6.3. Plating Out and Isolation

Xylose lysine desoxycholate (XLD, Oxoid Ltd., England) agar and brilliant green agar (BGA, Oxoid Ltd., England) plates were used for plating out and identification. A loop full of inoculums from each RV and MKTT broth cultures were plated onto XLD and BGA plates and incubated at 37 °C for 24 h. After incubation, the plates were examined for the presence of typical and suspect Salmonella colonies. Typical colonies of Salmonella grown on XLD agar have a black center and a lightly transparent zone of reddish color due to the color change of the media (ISO 6579-1:2017/Amd.1:2020(E)) while hydrogen sulfide negative variants grown on XLD agar were pink with a darker pink center. Lactose-positive Salmonella grown on XLD agar were yellow with or

without blackening. Typical colonies of Salmonella on BGA were pink, 1 mm to 2 mm in diameter, and change the color of medium to red (Quinn et al., 1999).

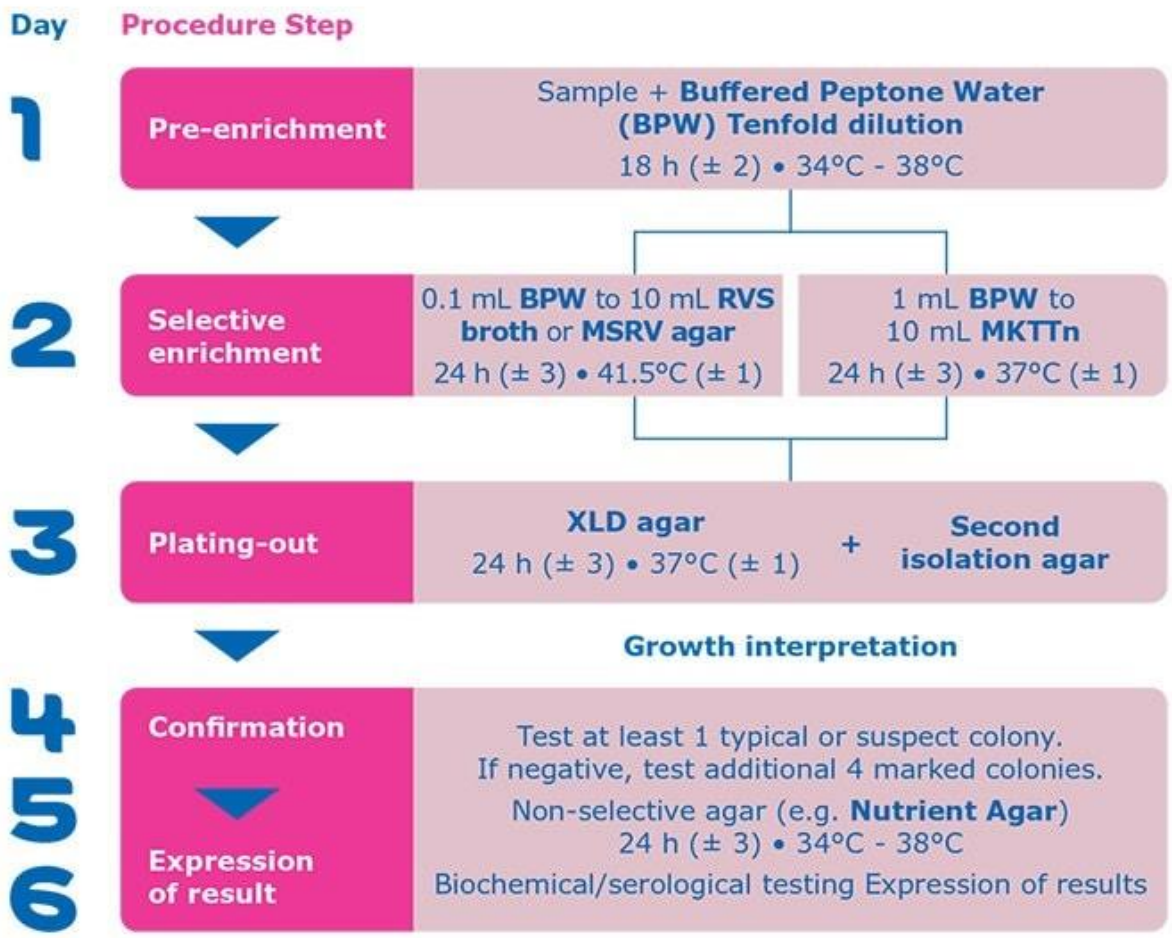


Figure 2. Salmonella species isolation procedures

Source: ISO 6579-1:2017/Amd.1:2020(E))

3.6.4 Biochemical Identification

The biochemical identification was done according to (Aziza *et al*., 2018) by using indole, Methyl red, Vogas-Proskaur, urease, citrate utilization, triple sugar iron (TSI), lysine decarboxylase and hydrogen sulphide production tests. All suspected Salmonella isolates were subjected to the following biochemical tests for confirmation: Triple Sugar Iron (TSI) test, Indole test, Citrate utilization test, Methyl red test, vogues Proskauer (VP) test, and urease test.

Colonies producing red slant (alkaline), with yellow butt (acid) on TSIA with blackening due to hydrogen sulphide (H_2S) production and gas production in butt, negative for Indole test, positive for Methyl red test (red broth culture), negative for urea hydrolysis

(yellow), positive for citrate utilization (deep blue slant), and negative for Voges-Proskauer (VP) test were considered to be Salmonella positive (ISO 6579, 2002; Quinn *et al.*, 2004) (ISO 6579-1:2017/Amd.1:2020(E) (2022). Fully isolated Bacteria were kept in BHI in 15% glycerol and stored at -20 OC till use.

3.6.5. DNA Extraction

Fifty one (51) Salmonella isolates that were stored in brain heart infusion (BHI) with 15% glycerol at -20 ◦C were revived using BHI broth and incubated for 12-18 h at 37 ◦C. The genomic and plasmid DNA of all strains used in this study was extracted using Thermo Fisher Scientific DNA extraction kit following the manufacturer's protocol (**Annex 3**).

3.6.6. Molecular Detection of Salmonella Species

Polymerase Chain Reaction (PCR) was conducted at the molecular Biology Laboratory of the college of Veterinary Sciences, Mekelle University. Forty two biochemically characterized Salmonella isolates that were purposively picked for the molecular detection of the Spy gene, which is peculiar to *Salmonella Kentucky, Salmonella Gallinarum* and *Salmonella typhimurium.*

The PCR protocol developed by Mohanapriya *et al*., 2023, was utilized. The primers summarized in Table 1 were used. Briefly, 25 µL reaction mixtures were prepared consisting of 12.5 µL of 2× DreamTaq Green PCR Master Mix (Thermo scientific). Three µL of primer mix, 5 µL of DNA template, 1 µL of DNTPs, 0.05 µL of polymerase, and nuclease-free water were added to make up to 25 µL (Annex 5). The reactions were carried out on a Thermo cycler. The program was as follows: initial denaturation at 95 ◦C for 5 min followed by 35 cycles of 95 ◦C for 1 min, 60 ◦C for 1 min, 72 ◦C for 2 min and a final extension of 72 ◦C for 7 min.

After amplification, the PCR products were analyzed by agarose gel electrophoresis and visualized under UV light. For gel preparation, a 1.5% agarose gel was made using 1x TAE (Tris-acetate-EDTA) buffer, following the manufacturer's guidelines (Annex 3a). A total of 10 µl of PCR product mixed with loading dye was loaded into the wells of the agarose gel. A molecular marker (3000-bp ladder) was included to determine the size of

the PCR fragments and agarose powder. The separated bands were visualized using a UV trans illuminator gel documentation system (Annex 2).

Table 1. Primers used in this study

Pathogen	Primer name	Forward (5'…3')	Reverse (5'…3')	Expected amplicon size (bp)
Salmonella Genus specific	INVA1/2	ACAGTGCTCGTTTACGACCTGAAT	AGACGACTGGTACTGATCGATAAT	244
Salmonella Kentucky	gly-F/R	TTCCAATTGAAACGAGTGCGG	ACTAACCGCTTGGGTTGTTGCTGT	170
Salmonella gallinarum	MS-GA-F/R	ATGGTGGGATTTGACGTGTT	CAGCGTTTAAGCTGCCAGAC	252 & 449
Salmonella tymuphrium	TYPHF/R	TTGTTCACTTTTTACCCCTGAA	CCCTGACAGCCGTTAGATATT	401

Source: Mohanapriya *et al*. (2023).

3.7. Questionnaire survey

3.7.1 Development of biosecurity scoring system

The Biosecurity toolkit of the Gent University was administered on small-and medium layer growers and producers. Efforts were made to include accessible respondents who were actively engaged in the poultry business those who participated in the microbiological examination. The general biosecurity scoring system includes groups of questions that are divided into several subcategories for external biosecurity factors including purchase of one-day-old chicks, purchase of laying hens, depopulation and transport of hens, transport of eggs, feed and water, removal of manure and carcasses, visitors and farmworkers, material supply, infrastructure and biological vectors, location of the farm. The internal biosecurity factors were Disease management, Cleaning and disinfection, Materials and measures between compartments and Egg management.

The answer to every question results in a score. This score can range from zero, indicating a total absence of the described biosecurity measures, to 100, indicating a full application of the described measures. The final overall biosecurity sore was the sum of the external and internal biosecurity scores which subdivided into different subcategory scores (Dewulf et al. 2018). The majority of the questions were taken from the Biocheck.UGentTM tool (https://biocheck.ugent.be/en). The online questionnaire was

expertly translated into the local Tigrigna by a professional translator, ensuring clarity and accessibility for both interviewers and interviewees. This adaptation was made to facilitate a smoother communication process and enhance the overall experience for all participants.

3.7.2. Data Collection Practices

A total of 28 farmers from small (15) and medium (13) scale layer poultry farms across different sub-cities in Mekelle participated in the study. Each participating farm was represented by a contact person, typically the farm manager or owner, who was interviewed during site visits. Most interviews were conducted in the farms' offices; however, some were held outside the gates to mitigate the risk of disease introduction.

The face-to-face interviews took place over a period from octeber2024 up to November 2024, ensuring a comprehensive assessment of biosecurity practices across diverse geographic locations. This approach allowed for a detailed understanding of the biosecurity measures implemented at each farm, as well as the challenges faced in maintaining these standards.

3.8. Data analysis

The raw data collected from the study were systematically arranged, organized, coded, and entered into an Excel spreadsheet using Microsoft® Office Excel 2007. Subsequent analysis was performed using SPSS version 27, focusing on descriptive statistics and employing chi-square tests to evaluate the data. The outcomes of these analyses were primarily expressed in terms of proportions, calculated as the number of samples testing positive for Salmonella relative to the total number of samples examined for the Sample level prevalence of Salmonella. Whereas the farm-level prevalence was calculated as the percentage of farms with one or more Salmonella culture positive samples among the total farms sampled. A chi-square test was used to assess the association between Salmonella occurrence and explanatory variables at the farm level and Sample level.

Additionally, Chi-square was utilized to assess the statistical significance of various risk factors with the result of the bacteriological and PCR tests. With the ultimate aim of quantifying the crude and adjusted odds ratio (OR), univariate and multivariable logistic

regression analyses were conducted, respectively. Statistical significance was declared whenever a p-value of less than 5% ($p < 0.05$) was attained. With regard to determining the effect of various risk factors on the basis of an OR 95% confidence interval, the significance of the statistical test was assumed whenever the confidence interval excluded one of its values.

Biochek. UGent, a risk-based scoring instrument created by the University of Ghent, was utilized to evaluate farm biosecurity. The data of the completed surveys were encoded online to generate ratings for each farm's biosecurity. The algorithm decoded and provided individual farm biosecurity scores across all categories and subcategories. Averages of scores were obtained for quantitative external biosecurity variables (purchase of pullets, transport of eggs, supply of feed and water, disposal of manure and corpses, entry of visitors and staff, supply of materials, infrastructure and biological vectors, and location of the farm) and internal biosecurity (disease management, cleaning, disinfection, equipment, and measures between compartments and egg management). The overall biosecurity scores in the various sub-categories were compared to the world average using the "Biocheck.UGent" test for a population average. Global averages were taken from all layer farms, which can be found on the "Biocheck.UGent" guide. The data were further compiled in Microsoft Office Excel 2016 for consolidation and statistical analysis through percentage and graphs for the external and internal biosecurity scores. In addition, a t-test was conducted to compare the total biosecurity scores of layer farms in the external and internal biosecurity subcategories to the global averages.

3.9. Ethical Clearance

This research is parts of Enhancing poultry biosecurity and health management through capacitating farmers, veterinarians and researchers in Central and Northern Ethiopia: towards improvement of poultry productivity (EPBHE)" VLIR SI project 2023. Ethical clearance for the study was given by the Animal research ethical review Committee of Mekelle University, College of Veterinary sciences in a letter dated on November 23, 2024 and reference number AEEC No. 30/2024. The significance of this research was evaluated from ethical perspectives, applicability, and originality technical competence point of view. A formal letter was written to poultry farms to get permission and

cooperation to conduct the study. Poultry farm owners were requested to participate in the study, and individual informed verbal consent was obtained from poultry in contact human subjects willing to participate in the study. Any information obtained from participants during the study was kept confidential.

4. RESULTS

4.1. Isolation and identification of Salmonella

A comprehensive bacteriological analysis was conducted on 250 cloacal swab samples, revealing that 34.8% (87/250) tested positive for the presence of the genus Salmonella. The characteristic appearance of Salmonella species on XLD agar was shown in Figure 2.

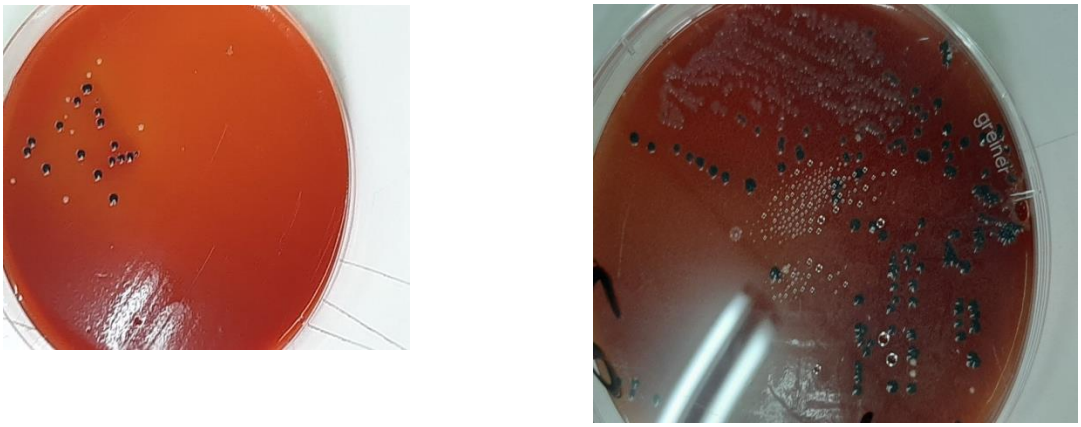


Figure 3. Salmonella growth on XLD agar

Salmonella species were isolated from 57.7% (16 out of 28) of the poultry farms examined. Salmonella species were not isolated from the other 12 poultry farms, specifically comprising 2 farms from Ayder, 3 from Hadnet, and 7 from Hawelti. The highest isolation rates were observed in farms located in Semien and Quiha, where 100% of samples tested positive for Salmonella isolates. This was followed by Ayder with an isolation rate of 60%. In contrast, the lowest isolation rates were recorded in Hawelti at 41.7% and Hadnet at 40% (Table 2).

Table 2. The isolation rate of Salmonella species based on bacteriological culture methods

Study site	No. of Farms	No. of samples	No. of Positive farms	No. of positive samples	Positive farms (%)	Positive samples (%)
Ayder	5	39	3	14	60	35.8
Hawelti	12	89	5	29	41.66	32.6
Semien	5	66	5	28	100	42.4
Hadnet	5	50	2	20	40	40
Quiha	1	6	1	2	100	33.3
Total	**28**	**250**	**16**	**87**	**57.14**	**34.8**

The occurrence of Salmonella species varied significantly based on flock size. Notably, Salmonella species were more frequently identified in poultry farms with medium flock sizes, exhibiting an occurrence rate of 42.1%. In contrast, the detection rate in farms with small flock sizes was lower, at 27.4%. The occurrence of Salmonella species was significantly higher in chickens older than 12 months, followed by those younger than 6 months and those aged between 6 to 12 months ($p < 0.021$). Additionally, the presence of other animals on the farm was associated with increased Salmonella isolation rates ($p < 0.022$), as well as practices involving on-farm waste disposal ($p < 0.001$). Furthermore, Salmonella isolation rates were notably higher in farms sourcing chickens from various suppliers within the last two years ($p < 0.001$) and in chickens reared in muddy floor housing conditions ($p < 0.022$). The occurrence of Salmonella species. Varied significantly based on flock size. Notably, Salmonella species were more frequently identified in poultry farms with medium flock sizes, exhibiting an occurrence rate of 72.8%. In contrast, the detection rate in farms with small flock sizes was lower, at 47.4 % ($p=0.0180$). Additionally, the presence of other animals on the farm was associated with increased Salmonella isolation rates ($p < 0.033$), as well as practices involving on-farm waste disposal (Table 3).

Table 3. Sample-level prevalence of Salmonella in poultry farms and associated factors in Mekelle

Variables	Category	No. of Samples Tested	No. of Positive samples	Percentage (%) with in the variable	$\chi 2$	P-value
Subcities	Ayder	39	14	35.9	4.288	0.758
	Hawelti	89	29	32.6		
	Semien	66	28	42.4		
	Hadnet	50	20	40		
	Queha	6	2	33.3		
Flock size	Small	124	42	27.4	5.907	0.015
	Medium	126	53	42.1		
Age (months)	< 6	82	23	28	7.727	o.o21
	6-12	57	15	26.3		
	>12	111	57	51.4		
Breed	Bovans brown	234	82	35	0.095	0.758
	Saso	16	5	31.3		
Waste disposal practices	On farm waste disposal	194	83	42.8	24.328	0.001
	Off farm waste disposal	56	4	7.1		
Floor house	Cement	130	38	29.2	3.705	0.054
	Muddy	120	49	40.8		
Presence of other animals	Yes	35	18	51.4	4.96	0.022
	No	215	69	32.1		
Rodent control	Yes	37	12	34.4	0.64	0.743
	No	213	75	35		
All in/all out	Yes	208	69	33.3	1.41	0.229
	No	42	18	42.9		
Wild bird control	Yes	129	43	33.3	0.253	0.615
	No	121	44	36.4		
Sources of chicken	same supplier	137	34	24.8	13.312	0.001
	different suppliers	113	53	46.9		

The analyses have identified significant associations between various factors and Salmonella detection in poultry samples. Key variables considered in the multivariable regression model included on-farm waste disposal practices, the sources of chicken supply over the past two years, the presence of other animal species on farms, chicken age, and flock size. These variables were considered for multivariable regression

modeling. Findings from the multivariable logistic model identified are summarized in Table 4. On-farm waste disposal practice was associated with an increased odds ratio of the birds being positive for *Salmonella* (crude odds ratio (COR) = 2.10; 95 % (1.11 -3.96) 0.022. The odds of *Salmonella* positivity was significantly higher in farms that the age birds greater than 12 months (AOR =2.37; 95% CI: 1.48 -3.79) than in farms that their age 6- 12 months.

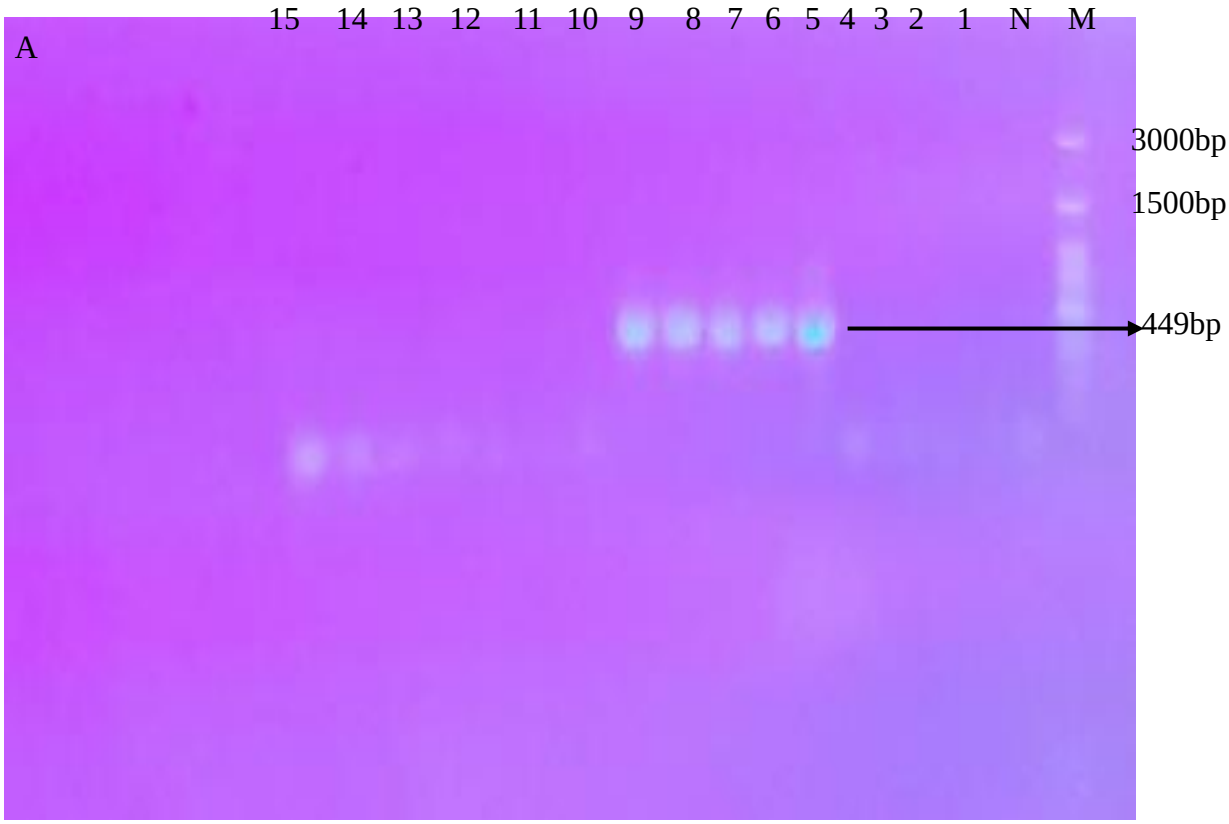
Table 4. Summary of findings from logistic regression showing association of sample-level Salmonella positivity

Selected factors	**Categories**	**Total No. of sample tested**	**Salmonella positive (%)**	**Univarable COR (95% CI) p-value**	**Multivariable AOR (95% CI) p-value**
Poultry farm's waste disposal practices	On-farm waste disposal	194	83(48.8%)	2.10(1.11 - 3.96) 0.022	---------
	Off -farm waste disposal	56	4(7.1%)	Ref.	
Sources of chicken supply over the past two years	Received from different suppliers	137	34(24.8%)	2.38(1.4 - 4.04) 0.001	----------
	Received from same suppliers	113	53(46.9%)	Ref.	----------
Presence of other animal species on farms	Yes	25	18(51.4%)	1.28(0.61 - 2.64)0.	----------
	No	215	69(32.1%)	Ref.	
Age of the chickens	>12 months	111	57(51.4%)	3.75(1.75 - 7.13) 0.000	2.37(1.48 - 3.79) 0.000
	6-12 months	57	15(26.3%)	Ref.	

*COR= Crude Odd Ratio; AOR = Adjusted Crude Ratio

4.2. Molecular characterization of isolates

Among the isolates, 51 that showed positive growth on XLD agar and characterized as *Salmonella species* through biochemical tests were further tested by conventional PCR with specific primers. Agarose gel electrophoresis showed that 82.4% (42 out of 51) exhibited successful amplification with the INVA1/2. 45% (45 out of 51) of these were positive for *Salmonella gallinarum* using the MS-GA-F/R primers, 35 %(18out of 51) of these were positive for *Salmonella tymuphrium* with the TYPH F/R (Figure 4). However, less amplification with light band was observed for the gly-F/R primers of the *Salmonella Kentucky*. Further details of the isolates are summarized in Table 5.



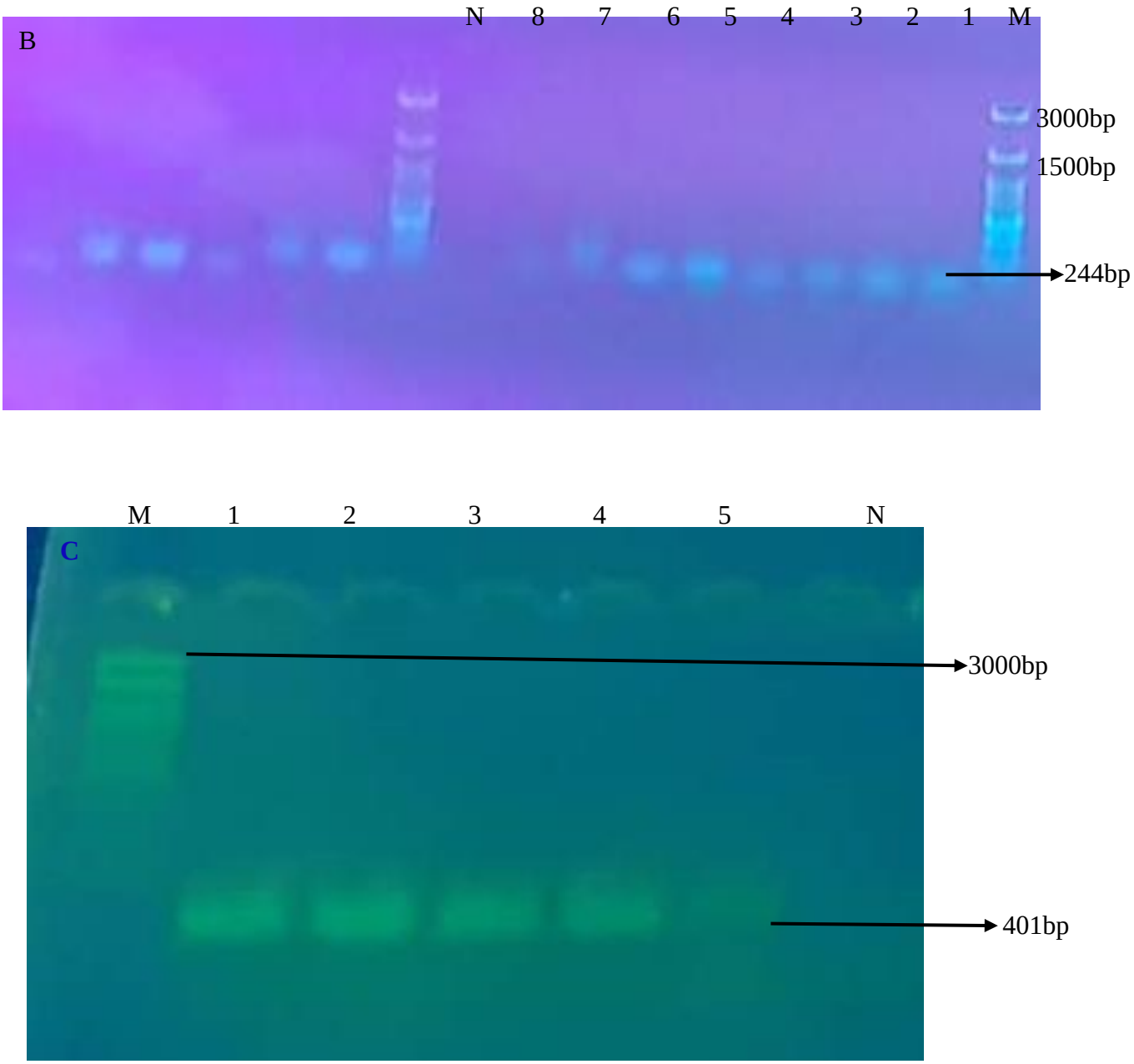


Figure 4. Agarose gel electrophoresis of the PCR products

Showing PCR amplified MS-GA-F/R (A), INVA1/2 (B) and TYPH F/R (C) fragments from poultry isolates of *Salmonella species* from poultry farms from Mekelle. M: Molecular marker 100–3,000 bp, lane 1– 15: test samples, lane N: negative control.

Table 5. Distribution of PCR amplified salmonella to its respected primers based on different risk factors (n=42 of 51)

Variables	Category	No. of Isolate	PCR INVA1/2 amplified %	Primers		
				MS-GA-F/R	TYPHF/R	gly-F/R
Age	<6months	25	25(100%)	17(68%)	8(32%)	
	6-12months	20	11 (55%)	2(10%)	8(40%)	3(15%)
	>12months	6	6(100%)	4(66.7%)	2(33.3%)	
	Total	51	42(82.4%)	23(45%)	18(35%)	
Flock	Small scales	32	32(100%)	18(56. %)	14(43.8%)	3(9.4%)
	Medium scales	19	10(52.6%)	5(26%)	4(21%)	
	Total	51	42(82.4%)	23(45%)	18(35%)	
Breed	Bovans	46	37(80.4%)	18(56. %)	18(39%)	3(5.5%)35
	Saso	5	5(100%)	5(26%)	--	
	Total	51	42(82.4%)	23(45%)	18(35%)	

4.3. Biosecurity measures using the Biocheck.UGent tool

The overall biosecurity score for layer farms in Mekelle is 57.86%, with the scores of 48.54% for external biosecurity and 67.11% for internal biosecurity. Among the external biosecurity subcategories, the highest mean scores were recorded for Infrastructure and biological vectors (mean = 70.11), Purchase of laying hens (mean = 69.29), Purchase of one-day-old chicks (mean = 61.63). Material supply (mean = 61.43) and Location of the farm (mean = 61.11). Conversely, the lowest score was noted in the category of Removal of manure and carcasses and Depopulation and transport of hens which had a mean of only 15.32, and 18.64 (Table 6). The external biosecurity score was significantly lower than the global average (48.54 versus 66; $p < 0.0148$). In terms of internal biosecurity, the subcategories of Disease Management and Cleaning and Disinfection achieved the highest mean scores, indicating effective practices in these areas (Table 6).

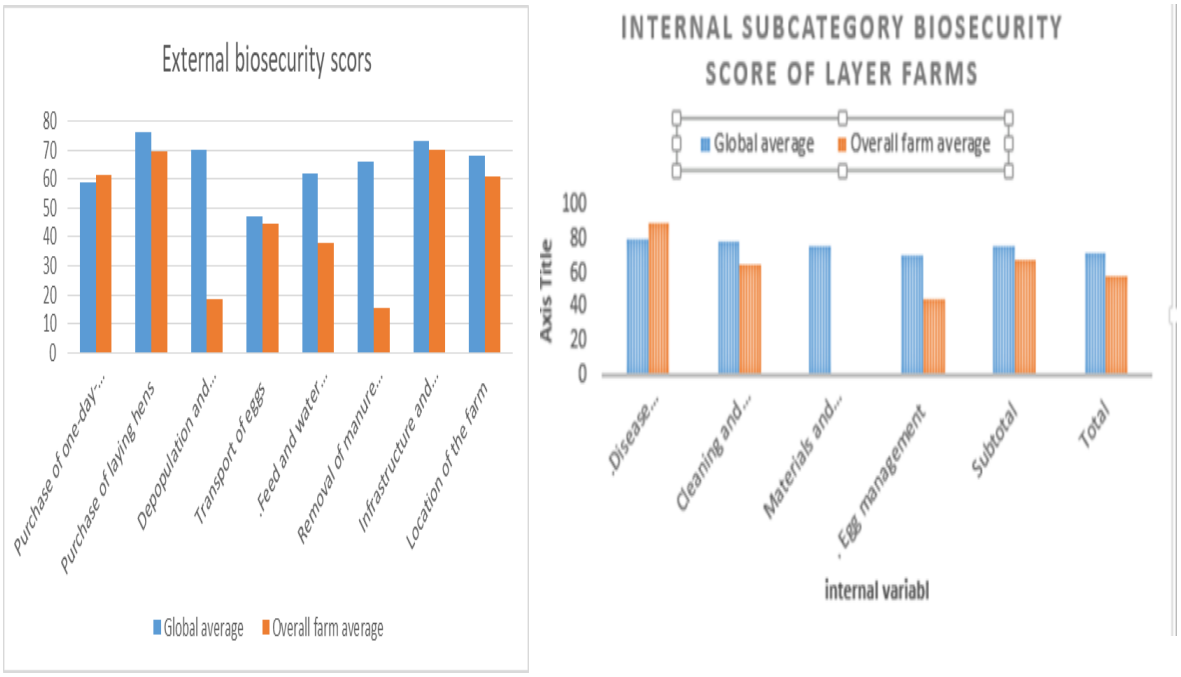


Figure 5. External and internal Biosecurity score of layer farms in Mekelle

Table 6 shows a t-test comparing the global and overall average scores for the various subcategories of biosecurity. Among the external biosecurity subcategories assessed, layer farms in this study achieved the highest mean scores in the following areas: Infrastructure and biological vectors (mean = 70.11), Purchase of laying hens (mean = 69.29), Purchase of one-day-old chicks (mean = 61.63), Material supply (mean = 61.43), and Location of the farm (mean = 61.11). In terms of internal biosecurity, the subcategories of Disease Management and Cleaning and Disinfection achieved the highest mean scores, indicating effective practices in these areas.

Table 6. t-test analysis of the overall average scores of layer farms for the different subcategories of biosecurity and global averages

Subcategory	**Global average**	**Overall farm average**	**p-value**
External biosecurity			
Purchase of one-day-old chicks	59	61.63	0.6741
Purchase of laying hens	76	69.29	0.1481
Depopulation and transport of hens	70	18.64	0.1299
Transport of eggs	47	44.36	0.0024
Feed and water management	62	37.68	0.0471
Removal of manure and carcasses	66	15.32	0.1822
Visitors and farmworkers management	73	53.79	0.0381
Material supply	75	61.43	0.0743
Infrastructure and biological vectors	73	70.11	0.3799
Location of the farm	68	61.11	0.0481
Subtotal	**66**	**48.54**	**0.01484**
Internal biosecurity			
Disease management	79	88.75	0.0540
Cleaning and disinfection	78	65	0.2047
Materials and measures between compartments	75	--------	0.170
Egg management	70	44.71	0.188
Subtotal	76	67.11	0.2329
Total	**71**	**57.86**	**0.0805**

Majority (64.3%) of the poultry farms purchased day-old chicks from the same supplier. Moreover, 53.6% of the farm owners didn't receive directly from the supplier. The routine practices in poultry farms that affect the biosecurity measures are summarized as below (Table 7).

Table 7. Routines practices for on Purchase of (one-day-old chicks, laying hens), Depopulation and transport of hens

Biosecurity Measures	Responses	Number of Farms	%
Purchase of one-day-old chicks			
The One-day-old chicks have always been bought from the same original source over the last two years.	Always the same supplier	18	64.3
	Sometimes a different supplier	10	35.7
First delivered at your farm	Always	13	46.4
	Sometimes	5	53.6
Transport vehicles are cleaned and disinfected before one-day-old chicks are loaded	Always	19	67.9
	Sometimes	9	32
Purchase of laying hens			
The laying hens (during the last 2 years) always bought from the same original source	Always the same supplier	20	71.4
	Sometimes a different supplier	8	28.6
Requested need to proof to ensure that the sanitary statute and health management	Yes	26	92.9
	No	2	7.
The bought laying hens are first delivered to your farm	Always	8	28.6
	Sometimes	20	71.4
Cleaned and disinfected before the laying hens are loaded	Always	8	28
	Sometimes	20	72
Depopulation and transport of hens			
Transport vehicle for poultry empty on arrival at the farm	Always	11	39.3
	Sometimes	15	53.6
The transport vehicle is for poultry free from visible dirt on arrival at the farm	Always	20	71.4
	Sometimes	8	28.6
the transport vehicle for poultry is cleaned and disinfected on arrival at the farm	Always	14	50
	Sometimes	12	42.9
	Never	1	3.6

Good routine practices that positively contribute to proper biosecurity measures were no access to drivers to egg facilities in the farm (89.3%), selling of eggs outside the farm (71.4%), storage of litter outside the farm (100%), presence of farm specific clothes (78.6%), and hand washing, disinfection during farm entry (71.4%) and most employees working only to a given farm (92.9%). On the other hand, the routine practices that negatively affect biosecurity measures were lack of clear demarcation between the clean and dirty parts of the farms (64.3%), lack of a separate room for morbid material (92%), and farms open to many visitors (53.9%) (Table 8).

Table 8. Routines on Transport of eggs, feeding and watering management, and Removal of manure and carcasses, Visitors and farmworkers

Biosecurity Measures	Responses	No, of Farms	%
Transport of eggs			
The driver does have access to the egg facilities of the farm	Yes, but only to the specific storeroom	1	3.6
	Yes, the driver has access to both the egg room and specific storeroom	2	7.1
	No, the driver doesn't have access at all	25	89.3
The eggs are being sold at the farm	Yes	8	28.6
	No	20	71.4
Feed and water			
the clean and dirty area of the farm site are clearly separated	Yes	10	35.7
	No	18	64.3
The feeding company can fill up the silos/deliver feed without entering the clean area	Yes	9	32
	Only some of them	2	42
	No	7	25
The feeding company fill up the silos per a year	Less than 20 times a year	2	7.1
	Between 20 and 35 times a year	4	14.3
	More than 35 times a year	22	78.6
Removal of manure and carcasses			
The manure being stored on the farm	Yes		
	No	28	100
The manure was kept in a container that was completely sealed.	Yes		
	No	28	100
The manure was taken out and properly disposed of.	Yes	2	7.
	No	26	92.9
The carcass storage in separate room	Yes	3	8
	No	25	92
The carcasses was manipulated with gloves	Always	3	10.7
	Sometimes	16	57.
	Never	12	42.9
cleaned and disinfected after removal of dead birds	Always	10	35.7
	Sometimes	15	53.6
	Never	3	10.7
Visitors and farmworkers			
visitors obliged to notify you his presence	Yes	25	89.3
	No	3	10.7
Presence of farm specific clothes	Yes	22	78.6
	No	6	21.4
Presence of farm specific shoes/overshoes	Yes	22	78.6
	No	6	21.4
Hand washing and disinfection during farm entry	Yes	20	71.4
	No	8	28.6
Employees working in different farms	Yes	2	7.2
	No	26	92.9
Access visitors times per year	Access is never granted	1	3.6
	Access is granted, but less than 12 times a year	12	42.9
	Access is granted more than 12 times a year	15	53.9

Other practices that affect biosecurity measures negatively include sharing of materials among farms (53.6%), exposure of chickens outside the farm (89.3%), entry of wild birds and vermin to the farm (89.3%), lack of adequate distance between farms (78.6%), and spread of manure within 500 meters (92.9%) (Table 9).

Table 9. Routines on Material Sharing, Farm Infrastructure and Biological Factors

Biosecurity Measures	Responses	No of Farms	%
Material supply			
Material being shared	Yes	15	53.6
with other farms	No	13	46.4
measures taken to material after receiving	Yes	13	46.4
prior usage	No	15	53.6
Infrastructure and biological vectors			
Access of chickens to	Yes	25	89.3
the outside (open air)	No	3	10.7
outdoor area enclosed with nets on all sides	Yes	26	92.9
	No	2	7.1
Access of wild birds and vermin to the	Yes	26	92.9
farm	No	2	7.1
farm fenced	Yes, it's completely fenced	26	92.9
	It's only partially fenced	2	7.1
	No		
paved and clean were outside of the farm	Yes, it's completely fenced	25	89.3
(around the walls)	It's only partially fenced	3	10.7
	No		
problem of vermin (i.e. rats, mice, etc.) at	Often	13	46.4
the farm	Sometimes	15	53.6
	Never		
Rodent control program	Yes, a professional pest control company has been hired	2	7.1
	Yes, I have established my own pest control	23	82
	No	3	10.7
	Never	12	42.9
Access of pet animals	Yes	8	28.6
(cats and dogs)	No	20	71.4
kept other animals on the same farm site	Yes	3	10.7
	No	25	89.3
Location of the farm			
Approximate distance	Less than 500 meters (Less than 0.3 miles)	22	78.6
from nearest poultry			
farm	Between 500 m and 1 km (0.3 - 0.6 miles)	4	14.7
	More than 1 kilometer (more than 0.6 miles)	2	7.1
stagnant or running water within a 1-	Yes	2	7.1
kilometre radius	No	26	92.9
manure spread within a 500-metre	Often	2	7.1
	Sometimes	26	92.9
	Never		

The routines on disease management practices and cleaning and disinfection practices are summarized in Table 10. The rewarding practices are daily health check of birds (96.4%), accessibility of professionals for health monitoring (85.7%), and optimal removal time for dead bird (100%), farming of similar bird ages per farm (75%), and cleaning of poultry houses after each production cycle (87%) (Table 10).

Table 10. Routines on Disease Management Practices and Cleaning and Disinfection Practices

Biosecurity Measures	**Responses**	**No, of Farms**	**%**
Disease management			
health checked on a daily	Yes	27	96.4
	No	1	3.6
Professional help for health status monitory	Yes	24	85.7
	No	4	14.3
Removal of dead birds per day	Twice or more a day	28	100
	Once a day		
	Less frequent than once a day		
Present different age categories	within one poultry house	5	17.9
	sorted by poultry house	3	7. 1
	No	21	75
Inspection from young to older birds	Yes	24	85.7
	No	2	7
Cleaning and disinfection			
Cleaned the poultry houses after each production cycle	Yes, dry and wet cleaning	23	. 1
	Yes; however, only dry cleaning	5	17.9
	No		
Pre-soaked with water before beginning the cleaning process.	Always	21	75
	Sometimes	7	25
	Never		
Detergent mixed with water during the cleaning process.	Always	21	75
	Sometimes	5	17.9
	Never	1	3.6
The poultry houses are disinfected following each production cycle	Always	20	71.4
	Sometimes	6	21.4
	Never	2	7. 1

5. DISCUSSION

The farm-level prevalence of Salmonella was 57.1%. The current study's prevalence rate of farms was higher than those reported in other regions of Ethiopia, which include 36.2% in Addis Ababa (Akalu *et al*., 2024), 28.8% in Adama and Modjo towns (Dagnew *et al*., 2020), and 14.6% in central Ethiopia (Eguale, 2018). These variations underscore the significant regional differences in Salmonella prevalence, suggesting that local factors may play a crucial role in influencing infection rates. This may be due to risk factors such as the source and quality of feed, interactions with other farms, the specific breeds of chickens raised, and the management practices employed in poultry care (Dagnew *et al.,* 2020).

The current study reports a Salmonella prevalence in poultry farms in Mekelle City, which is lower than the rates observed in other regions, such as 73.02% in Debire Birhan (Ayela, 2020). However, this finding was somewhat consistent with documented prevalence rates of 50.6% in Ethiopia (Waktole *et al.*, 2024), 56.5% in Pakistan (Ayesha Khan, 2014), and 55% in Nepal (Sharma et al., 2021). These discrepancies highlight the substantial influence of geographic, environmental, and management factors on Salmonella contamination levels across different poultry farming systems worldwide.

Salmonella detection was significantly higher in farms utilizing on-farm waste disposal methods compared to those employing off-farm disposal practices ($p = 0.001$). This suggests that on-farm waste may act as a source of contamination for chickens, facilitating the potential for on-farm transmission of Salmonella. Such results are consistent with previous findings from various African countries that have established a correlation between waste management practices and Salmonella prevalence in poultry farming (Abdi *et al.,* 2017; Andoh *et al.,* 2016). This highlights the need for improved waste management strategies to reduce contamination risks and enhance biosecurity measures within poultry operations. Effective waste management strategies, including safe disposal methods and thorough cleaning protocols, are essential to mitigate these risks and enhance biosecurity on poultry farms.

The sample-level prevalence of Salmonella reported in the current study is significantly higher than findings from similar investigations across Ethiopia. For instance, a study in central Ethiopia documented a prevalence of 14.4% (Waktole *et al*., 2024), while another

in southern Ethiopia found a prevalence of 16.7% (Abdi *et al*., 2017). In Addis Ababa, samples taken from poultry farms showed a prevalence of 6.4% (Akalu *et al*., 2024). Additionally, research conducted in and around Arba Minch town, Gamo Zone, reported a prevalence of 5.38% (Abayneh *et a*l., 2023). Comparatively, studies from other countries have also shown notable lower prevalence rates. For example, Salmonella prevalence per a given farm was reported at 15.9% in Nigeria (Jibril *et al*., 2020) and 16.2% in Uganda (Kakooza *et al.,* 2021). These findings underscore the regional variations in Salmonella prevalence and highlight the ongoing public health challenges associated with this pathogen in poultry farming. This finding was relatively consistent with previous research conducted in Mekelle, Tigray, Ethiopia, which reported a Salmonella prevalence of 32.8% by (Berhe *et al.,* 2012). Similarly, a study from Bangladesh revealed a prevalence of 31.25% (Mridha *et al*., 2020).

On the contrary, the sample level prevalence of Salmonella species was lower than previous studies carried out in the USA at 38.8 % (Alali *et al*., 2021) and in India at 55% (Sharma *et al.,* 2021). The variations in Salmonella prevalence observed in this study compared to previous findings can be attributed to several factors, including differences in isolation methods, the types and quantities of samples collected, geographic location, and the specific breeds of birds studied, particularly the more exotic Bovans Brown. Additionally, the types of poultry production systems, specifically focusing on small and medium-scale commercial laying hens, play a crucial role in these discrepancies (Abayneh *et al.,* 2023, Waktole *et al.,* 2024, Mridha *et al*., 2020).

A notable finding from the current research has shown a significant relationship between the age of hens and their likelihood of testing positive for Salmonella species. The odds of testing positive for the bacterium are 3.75 times higher for chickens older than 12 months compared to those aged 6-12 months. This indicates a significant association between age and the likelihood of testing positive. The odds ratio has a 95% confidence interval of (1.75 - 7.13), The adjusted odds ratio of 2.37 (1.48 - 3.79) with a p-value of 0.000 indicates that even after controlling for potential confounding variables, older hens still have more than twice the odds of testing positive compared to younger hens.

Hens older than 83 weeks have 1.63 times higher odds of being positive for this bacteria compared to younger birds under 39 weeks old, with a 95% credible interval ranging

from 1.35 to 2.00(Sharma *et al.,* 2021: Namata *et al.*, 2008) . The heightened risk of Salmonella positivity in older hens may stem from several interconnected factors. As hens age, there is often an increased bacterial load in their environment, resulting from the accumulation of Salmonella. Additionally, older birds have had more opportunities for exposure to the bacteria over time and a decline in immune response with age, and environmental (Sharma *et al*., 2021). In general, layers' physiological stress during egg-laying and molting may be the cause of Salmonella in adult chickens since it lowers their immune response and makes them more vulnerable than young chickens (Abunna, *et al.,* 2016).

The prevalence of salmonella differed significantly ($p < 0.05$) among farms with varying stock sizes. This was in agreement to various authors (Eguale, 2018; Dagnew *et al*., 2020: Akalu *et al.,* 2024). This was mostly because of increased bird numbers and the difficulties in overseeing bigger flocks. Management challenges and opportunities for cross-contamination are more prevalent in larger farming operations (Eguale, 2018, and Sharma *et al*., 2021). The number of isolates from small flock farms may have been caused by their lax biosecurity procedures, which are uncommon in affluent nations with recognized programs to carry out control measures but are absent in developing nations such as Ethiopia (Jibril et al., 2020, Haque *et al*., 2021).

Previous research has shown that inadequate management and poor biosecurity can lead to higher Salmonella contamination levels (Akalu *et al.,* 2024; Abayneh *et al.,* 2023). In contrast to findings from other regions where environmental factors significantly impact Salmonella prevalence, this study highlights the crucial role of management practices and biosecurity measures in influencing infection rates.

The results underscore the need for enhanced biosecurity protocols, improved waste management practices, and careful sourcing of poultry to effectively mitigate the risk of salmonellosis in commercial laying hen operations. Therefore, implementing robust biosecurity measures is essential for reducing Salmonella prevalence and ensuring the health of poultry flocks.

A significant correlation has been observed between Salmonella prevalence and the sources of chickens supplied to farms. Specifically, farms that received chickens from different suppliers showed a marked increase in Salmonella positivity ($p = 0.001$)

compared to those sourcing from the same suppliers. This finding suggests that introducing chickens from various farms may increase the risk of bringing Salmonella into new areas, potentially contaminating local flocks. This result aligns with similar studies conducted in South Ethiopia, Uganda, and Addis Ababa (Abdi *et al*., 2017, Andoh *et al.,* 2016, Akalu *et al.,* 2024).

In this study, the Salmonella genus was successfully amplified in 82.4% of the samples tested, with 42 out of 51 samples yielding positive results using the INVA1/2 primers. This result highlights the effectiveness of conventional PCR as a diagnostic tool for identifying Salmonella species, which is crucial for monitoring and controlling foodborne pathogens. The choice to target the INVA gene for amplification is specific to the Salmonella genus, making it a reliable marker for detection. The invA gene, which plays a crucial role in the pathogenicity of Salmonella, has been extensively validated as a target for molecular detection methods. Studies have shown that PCR assays targeting this gene can provide rapid and accurate results, making them invaluable tools in food safety and public health surveillance (Hernandez *et al*., 2018; Joseph *et al.,* 2016). Some studies indicate that certain non-typhoidal Salmonella serotypes might exhibit variability in their genetic makeup, leading to false negatives in PCR assays targeting specific genes like invA, may interfere with the PCR reaction, Technical Limitations. The detection rate of Salmonella Typhimurium at 35% in the current study aligns closely with findings from several other regions, highlighting the prevalence of this serovar in poultry. Specifically, this detection rate is similar to the 46.4% reported in South Africa (Olobatoke and Mulugeta, 2015), 43.35% in Morocco (Abdellah et al., 2009), and 40% in Greece (Adhikari et al., 2018). Conversely, it is notably lower than the 50% prevalence reported in Central Ethiopia (Waktole et al., 2024) and the 48.9% found in Vietnam (Luu et al., 2006).

The variations in Salmonella detection rates was could be due to several factors, including sampling techniques, geographic factors, and methodological differences. Each of these factors plays a critical role in influencing the prevalence and detection of Salmonella in poultry, as evidenced by various studies like (Jibril et al., 2020)

The 45% detection of Salmonella Gallinarum by PCR test in the present study was found to slight higher than to a study conducted in Bangladesh reported a detection rate of approximately 25.80% for Salmonella Gallinarum among 765 samples collected from commercial layer flocks (Haque et al., 2021) and much higher than In a study involving integrated crop-livestock farms, S. Pullorum was detected in 2.7% of samples (Julianingsih et al., 2024). Similarly higher than to globe as the A systematic review and meta-analysis covering global data from 1945 to 2021 indicated an overall prevalence of S. Gallinarum at 8.54%(Zhou et al., 2022). By amplifying particular DNA fragments, the widely used methods for detecting and identifying bacteria are agarose gel electrophoresis and polymerase chain reaction (PCR). These methods can speed up detection and offer a high specificity. False negative results could arise from PCR failing to achieve the detection limit for target fragments in small amounts. Consequently, more sensitive detection techniques like gene sequencing and quantitative PCR (qPCR) are required for further testing (Heymans *et al.,* 2018).

The biosecurity assessment of layer farms in Mekelle reveals concerning results, with overall scores for both external and internal biosecurity significantly lower than various parts of the world. Farms in Central Luzon, Philippines, achieved an overall biosecurity score of 65.9%, with external and internal scores of 63.3% and 71.9%, respectively (Tanquilut *et al.,* 2020). Similarly, in Sierra Leone, the average biosecurity ratings were even higher, with an overall score of 67.2%, including 64.4% for external measures and 70% for internal practices (Sesay, 2022). European conventional broiler production scoring greater differences in the mean overall biosecurity (70.9%) score were found. Compared to the European average, the observed mean external and internal biosecurity ratings were much lower (68 external and 76.6 internal) (Van Limbergen *et al*., 2018).

In contrast, The overall mean biosecurity score of poultry farms in Mekelle city, Ethiopia, was notably a bit higher than the biosecurity scores from Algeria, in Central Ethiopia and in Jimma town, Ethiopia, with an overall score of 54%, external score of 54%, and internal score of 45% (Alloui *et al*., 2021) . Similarly, a study in Central Ethiopia reported even lower scores: 43.1% overall, 40.7% for external biosecurity, and 52.2% for internal biosecurity (Waktole *et al.,* 2023). Similarly, a study of biosecurity score of poultry farms in Jimma town, Ethiopia, reported at 41.7% overall, at 44.9% for external

biosecurity, and at 31.6% for internal biosecurity (Kabeta *et al.*, 2024). The discrepancies in biosecurity scores across these regions may be attributed to factors such as farm type, production methods, and management systems.

Effective biosecurity measures are essential for preventing disease introduction and spread, particularly through vehicle movements. The challenges in implementing effective biosecurity measures on poultry farms arise from several critical factors, including a lack of access to clean water, inadequate waste disposal systems, insufficient cleaning supplies, unauthorized access to farm areas, poor control over the movement of people and animals, ineffective pest management strategies, and limitations in infrastructure (Schneider and Arndt, 2020). Recommendations emphasize the importance of cleaning and disinfecting transport vehicles before loading poultry to minimize disease outbreak risks linked to inter-farm vehicle traffic (Gelaude *et al*., 2014).).

In the assessment of external biosecurity measures, layer farms in this study achieved their highest mean scores in Infrastructure and biological vectors (mean = 70.11), Purchase of laying hens (mean = 69.29), Purchase of one-day-old chicks (mean = 61.63), Material supply (mean = 61.43), and Location of the farm (mean = 61.11). In contrast, farms in Sierra Leone reported significantly higher scores for external biosecurity, particularly in Location of the farm (mean = 92.4), Material supply (mean = 90.8), and Purchase of laying hens (mean = 84) (Sesay, 2022). Similarly, layer farms in Central Luzon, Philippines, noted high scores for Supply of materials (mean = 87.1) and Purchase of pullets (mean = 83.7) (Tanquilut *et al*., 2020). However, there are critical areas that require improvement across these farms. Specifically, Mekelle's layer farms need to enhance their practices related to the removal of manure and carcasses, as well as the depopulation and transportation of hens. This could be due to the challenges in implementing effective biosecurity measures often stem from economic limitations that restrict their ability to invest in critical areas such as infrastructure improvements, cleaning supplies of materials, and waste management systems.

Proximity of farms can increase the risk of disease transmission, as pathogens can easily spread between neighboring operations. Supplier diligence is also essential; farms that source birds from unreliable suppliers may inadvertently introduce diseases into their flocks (Morris, 2024). Over the past two years, 64.3% of farm owners have consistently

sourced their day-old chicks from the same supplier, but 53.6% of these chicks are not delivered directly to the farms. Many of the farms use transport methods lacking proper sanitation protocols, raising concerns about disease transmission among the chicks. Alarmingly, 100% of the farms had not conducted annual bacteriological analyses of their potable water, indicating a significant gap in water quality management. This finding contradicts sharply with a study in Central Luzon, Philippines, where 80% of farms had separate clean and dirty areas and 67.7% conducted annual bacteriological analyses (Tanquilut *et a*l., 2020).

Over the past two years, 71.4% of farmers have not changed their suppliers of laying hens. A significant 92.9% of farm owners request proof of sanitary status and health management practices when purchasing hens from other farms, which is crucial for maintaining biosecurity and flock health. When sourcing laying hens from Ethiochicken, only 28.6% of farmers report that the hens were always delivered to their farms first, while 71.4% said sometimes. This delivery rate is notably lower than in Central Luzon, Philippines, where 91% of farms disinfect delivery trucks before entering and 73% ensure first deliveries to their farms (Tanquilut *et al*., 2020). The greatest risk of introducing infectious diseases occurs when newly purchased animals remain in prolonged indirect contact with existing herds (Mee *et al.,* 2012). It is of high importance to limit the number of source herds as much as possible to avoid the introduction of certain infectious diseases (Gelaude *et al*., 2014).

The majority of the farms in this study severely violated the management of visitors and farmworkers, which led to a low level of implementation ($p < 0.05$) compared to the corresponding global average (53.79 in the study region vs. 73 globally). Visitors can potentially act as vectors for transmission of pathogenic agents in the farms (Lister, 2008).

Most farms (79%) indicated that a "zero-meter distance" refers to less than 500 meters from **n**eighborin**g** farms, with only 7% reporting distances greater than one kilometer. Consequently, land for poultry farms is typically allocated by the government through small and micro-enterprises. This situation contrasts sharply with findings from Sierra Leone, where 94.5% of layer farms had their nearest neighboring poultry farm located more than one kilometer away (Sesay, 2022). This results also quite lower the central

Ethiopia that found majority of the farms were located within less 500 meters of approximate distance from nearest poultry farm (56%) (Tadesse *et al*., 2024). Within a one-kilometer radius of their locations, 90% of layer farms reported the absence of stagnant or flowing water, which is a critical factor in preventing disease outbreaks. This finding aligns with studies conducted by Tsegaye *et al.* (2023) and Ismael (2021), which indicated that 86.9% and 70.45% of farms in the Arsi and East Showa zones, as well as in Bisheftu town, Oromia State, Ethiopia, were situated far from standing water sources. Danger of infectious disease transmission may be considerably decreased if two distinct chicken farms are at least 500 meters apart, ideally more than 1 kilometer (Van Steenwinkel *et al.*, 2011).

Disease management practices were implemented by 88.75% of layer farms, surpassing the global average of 79% (see Table 10). This internal biosecurity component score also exceeds the 52.2 reported in Central Ethiopia (Waktole *et al.*, 2023). Moreover, all layer farm owners or workers conducted daily checks and promptly removed dead animals, demonstrating a commitment to biosecurity. While 85% of farms received inspections from private veterinarians, only 35% performed postmortem examinations on dead birds to assess lesions. These findings align with previous studies conducted in Central Luzon, Philippines (Sesay, 2022; Tanquilut *et al.*, 2020).

Observations of cleaning and disinfection practices in layer poultry farms in Mekelle revealed that 82% of the farms effectively cleaned their poultry houses after each production cycle, aligning with previous reports indicating 74% adherence in Central Ethiopia (Waktole *et al*., 2023). Most of the farms (84%) soaked their poultry houses with water prior to cleaning, and 75% added detergent to the water during this process. Furthermore, 75% of the farms disinfected their poultry houses after each production cycle and allowed them to dry before commencing disinfection. Cleaning and disinfection practices are crucial for lowering pathogen levels on equipment, workers, and vehicles, thereby reducing disease transmission risks both between and within farms (Gosling, 2018).

6. CONCLUSION AND RECOMMENDATIONS

This study revealed a higher Salmonella prevalence of 57.1% at the farm level and 34.8% at the sample level. The study identified several significant associated factors with the presence of Salmonella species in the laying hens: Chickens received from different suppliers were more likely to be infected. Farms that housed other animals alongside chickens showed increased Salmonella prevalence. Poor waste management practices contributed to higher contamination rates. The high amplification rate (82.4%) of the INVA1/2 gene underscores the reliability of PCR as a diagnostic tool for identifying Salmonella species. The findings also indicate that while S. gallinarum is prevalent. Further optimization may be needed for others, as no and/or low amplification was observed for some primers used. The biosecurity assessment revealed an overall score of 57.86%, significantly below the global average of 66%. The findings underscore a pressing need for enhanced biosecurity protocols on layer farms in Mekelle, particularly focusing on external biosecurity measures to align more closely with global standards. This evaluation facilitated a more accurate evaluation of the dangers present on the farm and offered a common standard by which producers may attain and preserve a high level of biosecurity. Therefore, in line with the above concluding remarks, the following recommendations were forwarded:

- Strengthening Salmonella prevention and control efforts by enhancing biosecurity measures at poultry production facilities is critical.
- Future studies should focus on longitudinal research to evaluate the long-term effects of biosecurity interventions on reducing Salmonella prevalence.
- Providing training and resources to farm workers can help improve awareness and enhance biosecurity practices, fostering a healthier environment for poultry.
- Further research into the molecular characteristics of Salmonella isolates in the region is needed to better understand transmission patterns, identify circulating serotypes, and develop targeted control strategies.

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8. ANNEXES

Annex 1: Bacteriological media preparation for *Salmonella* isolation and identification

a. Buffered Peptone Water (Himedia, Mubai, India)

Composition	**g/l**
Enzymatic digest of casein	10.0
Sodium chloride	5.0
disodium phosphate dodecahydrate	9.0
potassium dihydrogen phosphate	9.0

Preparation:

Suspend 20.07 grams of dehydrated components in 1000 ml of distilled water. Mix well and heat (if necessary) to dissolve the medium completely. Distribute it into a universal bottle of suitable capacity to obtain the portions necessary for the test and sterilize in an autoclave at 1210C for 15 minutes.

b. Modified Rappaport Vassiliadis (HIMEDIA, Mumbai, India)

Composition	g/l
soya peptone	4.5
sodium chloride	8.00
potassium dihydrogen phosphate	0.60
dipotassium phosphate	0.40
magnesium chloride, hexahydrate	29.00
malachite green	0.036

Preparation:

Suspend 27.11 grams of the hydrated medium in 1000ml distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired into tubes and sterilize by autoclaving at 115 ℃ for 15 minutes. PH after sterilization: 5.2 + 0.2

c. Xylose Lysine Desoxycholate Agar (XLD) (New Mubai, India)

Composition	**g/l**
yeast extracts	3.0
l-lysine hydrochloric acid	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
L-Lysine hydrochloride	5.0
sodium chloride	5.0
sodium thiosulphate	6.8
ferric ammonium citrate	0.8
phenol red	0.08
Agar	15.0

Preparation:

Suspend 56.68 grams in one liter of distilled water. Heat with frequent agitation until the medium boils. **DO NOT OVER AUTOCLAVE**. Transfer immediately to a water bath at 50℃. pour into plates as soon as the medium has cooled. It is important to preparing large volumes which will cause prolonged heating.

d. Nutrient Agar (CM 0003, OXOID, Basingstoke, England)

Composition	**g/l**
peptic digest of animal tissue	5.00
sodium chloride	5.00
beef extract	1.5
yeast extract	1.5
Agar	15

Preparation:

Suspend 28 grams in 100ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 Ibs pressure (121℃) for 15 minutes. Mix well and pour into sterile Petri dishes. Final PH (at 25℃): 7.4 + 0.2.

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e. Triple Sugar Iron Agar (Himedia, Mubia, India)

Composition	**g/l**
meat extract	3.0
yeast extract	3.0
Peptone	20.0
sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
ferric citrate	0.3
sodium thiosulfate	0.3
phenol red	0.024
Agar	12.0

Preparation:

Suspend 64.52 grams in 1000ml of distilled water. Mix well and bring to boil to dissolve completely. Distribute in 20 ml tubes (8-10 ml/tubes) and sterilize by autoclaving at 121℃ for 25 minutes. Allow the set as slope with 2.5 cm butts. PH: 7.4 + 0.2 at 25℃.

f. Simon Citrate Agar (Himedia, Mubia, India)

Composition	**gms/l**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotasium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromo thymol blue	0.08
Agar	15

Preparation:

Suspend 24.28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired in tubes and sterilize by autoclaving at 1210C for 15 minutes.

g. MR-VP Medium (New Mumbai, India)

Composition	**grm/l**
pancreatic digest of casein	3.5
peptic digest of animal tissue; dextrose	5.00
dipotassium phosphate	5.00

Preparation:

Suspend 17.0 grams in one liter of distilled water. Heat to dissolve the medium completely (if necessary). Distribute in to test tubes 5ml amounts and sterilize by autoclaving at 15 Ibs pressure (121℃) for 15 minute

Reagent required for voges- proskauer reaction

α-Naphtanol, ethanolic solution, potassium hydroxide crystal, and distilled water

α-Naphtanol solution:

Composition	**Amount**
α-Naphtanol	5 grams
95 % ethano	100ml

Preparation:

Weight 5 gram α-Naphtanol crystal, dissolve in 250 volumetric flask containing 100 ml 95% ethanol and stored at +40C for about two to three weeks

Potassium Hydroxide Solution.

Composition	**Amount**
potassium hydroxide	40 grams
distilled water	100ml

Preparation:

Weight 40 gm potassium hydroxide and dissolve in 250 ml polyethylene bottle. Containing 100 ml distilled water and stir to dissolve the pellets and Keep the bottle in a cool water bath during preparation since the reaction procduce heat.

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h. Tryptone Soya broth Preparation (Oxoid Ltd, Hampshire, England)

Composition	**g/L**
pancreatic digestion of	17

casein	
Enzymatic digest of soya bean	3.0
sodium chloride	5
Dipotassium hydrogen phosphate	2.8
Glucose	2.5

Preparation:

Dissolve 30 gm of powder in 1 liter of distilled water as required and distribute to 5ml test tube of the final container. Sterilized by Autoclaving at1210c for 15 minutes. The broth was clear and yellow.

i. Lysine Decarboxylation Medium

Composition	**g/l**
Peptone	5.0
Beef Extract	5.0
Glucose	0.5
Bromcresol Purple	0.5
Cresol Red	5.0
Pyridoxal	5.0

Preparation:

Suspend 14 grams in a litter of distilled water. Heat to boiling to dissolve the medium completely. Dispense as desired in tubes and sterilize by autoclaving at 1210C for 15 minutes

Annex 2: Preparation of agarose gel, molecular marker, and Loading dye for PCR

a. Agarose gel (1.5%)

Composition:

TAE Buffer (1:49 ratio of concentrated stock TAE buffer and distilled water); agarose powder: 1.5 gm70

Preparation:

First prepare Tris Acetate EDTA (TAE) buffer by mixing 980 ml distilled water and 20 ml concentrated stock TAE buffer. Then weigh 1.5 gm agarose powder and dissolve in 200 ml TAE buffer in a 250 ml flask and melt the agaross in the microwave for around 8-12 minutes. Repeat the boiling until it becomes clear and let the solution cool to about 50-55°C. Seal the ends of the cas

ting tray with two lying of tape and place the combs in sealed gel casting tray. Pour on the gel try and wait for about 20 minutes until well solidified and carefully pull out the combs and remove the tape. Place the gel in the electrophoresis chamber and add enough TAE Buffer (about 2-3 mm of buffer over the gel).

b. Loading dye

Composition	**Concentration**
Loading buffer	6X
Gel Red	1000X

Procedure:

Mix 940 µl loading buffer and 60 µl Gel Red in 20 ml centrifuge tube by vortexing. Add 4 µl for 20 µl of PCR products, mix by pipeting and load 10 µl in to the Gel.

c. Molecular marker

Composition	**Amount**
loading buffer	475 µl
Gel Red	25 µl
PCR water	265 µl

Procedure:

Mix 475 µl loading buffer and 25 µl Gel Red by vortexing. Again mixing **265** µl PCR water, 59 µl pre-prepared mixture of loading buffer and Gel Red and 27 µl of DNA ladder by vortexing. Finally add add 10 µl in to the gel as a molecular marker

Kit contents

Table 1 PureLink™ Microbiome DNA Purification Kit (Cat. no. A29790, 50 reactions).

Component	Quantity	Storage
S1—Lysis Buffer	40 mL	15°C to 30°C
S2—Lysis Enhancer	5 mL	
S3—Cleanup Buffer[1]	12.5 mL	
S4—Binding Buffer	45 mL	
S5—Wash Buffer Concentrate[2]	13 mL	
S6—Elution Buffer	5 mL	

Sources. Thermo fisher, SCIENTIFIC

Important procedural guidelines

Sample input requirements and handling

• Collect samples according to your laboratory guidelines and Experimental needs.

• Recommended input amount:

Table 2

Sample type	Amount
Microbial culture	0.2–2 mL
Transport media[1]	0.2–5 mL

Sources. Thermo fisher, SCIENTIFIC

Swab removed; squeeze swab against the side of the tube before removal to ensure maximal release of cells into the media.

For either sample type, volumes as low as 0.2 mL can be used. For 0.2-mL samples, you may add the sample directly to 600 µL of S1—Lysis Buffer, instead of centrifuging and resuspending the microbial pellet in 800 µL of S1—Lysis Buffer.

• Ensure that samples are mixed thoroughly with S1—Lysis Buffer and S2—Lysis Enhancer to create a homogenous sample.

Annex 3: **Methods:** PureLink™ Microbiome DNA Purification Kit

Purification of high-quality microbial DNA from microbial culture and transport media samples

Perform the procedure at room temperature (20–25°C), unless otherwise indicated

1 Prepare the lysate

A. Centrifuge the sample at 14,000 × g for 10 minutes to pellet the microorganisms.

Samples larger than 5 mL may require longer centrifugation times for optimal recovery of microorganisms.

Use an appropriately sized centrifuge tube; do not use a Bead Tube.

B. Carefully remove the supernatant and discard.

Avoid disturbing the microbial pellet during this step. Loss of pellet will result in decreased yield.

C. Suspend the microbial pellet in 800 µL of S1—Lysis Buffer, pipet up and down or vortex to

resuspend, then transfer the sample to the Bead Tube.

D. Add 100 µL of S2—Lysis Enhancer, cap securely, and vortex briefly.

E. Incubate at 65°C for 10 minutes.

F. Homogenize by bead beating for 10 minutes at maximum speed on the vortex mixer.

Use the hands-free adapter and horizontal agitation.

G. Centrifuge at 14,000 × g for 2 minutes.

H. Transfer up to 500 µL of the supernatant to a clean microcentrifuge tube, avoiding the bead pellet and any debris.

2. Bind the DNA to the column

. Add 900 µL of S4—Binding Buffer, and vortex briefly.

B. load 700 µL of the sample mixture onto a spin column-tube assembly and centrifuge at 14,000 × g for 1 minute.

C. Discard the flow-through, and repeat step 2b with the remaining sample mixture

3. Wash and elute the DNA

a. Place the spin column in a clean collection tube, add 500 µL of S5—Wash Buffer, then centrifuge

the spin column-tube assembly at 14,000 × g for 1 minute.

b. Discard the flow-through, then centrifuge the spin column-tube assembly at 14,000 × g for

30 seconds.

The second centrifugation optimizes removal of S5—Wash Buffer, which could interfere with downstream applications.

c. Place the spin column in a clean tube, add 100 µL of S6—Elution Buffer, then incubate at room

Temperature for 1 minute.

d. Centrifuge the spin column-tube assembly at 14,000 × g for 1 minute, then discard the column.

The purified DNA is in the tube.

The DNA is ready for immediate use. Alternatively, store the purified DNA:

• At 4°C for up to 1 week.

• At –20°C for long-term storage.

Annex 4. The PCR reaction was set up using specific primers targeting the invA gene of Salmonella. The reaction mixture contained DNA template from the swabs, Taq polymerase, dNTPs, buffer, and the forward and reverse primers.

The PCR cycling conditions included an

Initial denaturation at 95°C for 5 minutes,

Followed by 35 cycles of denaturation at 95°C for 30 seconds,

Annealing at 55 °C for 30 seconds, and

Extension at 72°C for 2 minutes.

A final extension step was carried out at 72°C for 7 minutes.

Incubtion step at 4°C and the PCR product was used immediately or incubated at 4°C .

After the PCR reaction, the products were analyzed by agarose gel electrophoresis to confirm the presence of a specific amplicon indicative of Salmonella contamination in the poultry samples.

PCR component volume (µl)

10x reaction buffer = 2.5

dNTPs =0.5

Primer F =0.5

Primer R = 0.5

Templet = 1

Taq DNA polymerase =0.5

dd H_2O = 19.5

total volume = 25

Annex 7. Pictures showing poultry house and handling sample collections and processing in laboratory






A poultry rearing in the muddy floors house

Mekelle University

Animal Ethics and Experimentation Committee (AEEC) Clearance Letter

Date: November 23, 2024

To: Aregawi Gerekidan, Principal Investigator (PI)

Mekelle

Subject: Notification of AEEC decision on your research proposal

AEEC No: AEEC 30/2024

Protocol (Title of the study): Biosecurity Practices and Molecular Epidemiology of *Salmonella Species* in Small to Medium-Scale Commercial Layer Farms in Mekelle City, Northern Ethiopia

Dear PI,

The aforementioned research proposal which was submitted by you for animal ethics and use clearance letter has been reviewed by the Animal Ethics and Experimentation Committee (AEEC).

The AEEC has discussed and examined the research proposal in detail from animal ethics and use principles and values perspective.

Finally, the AEEC has (**approved**, approved with modification or withhold approval) your research proposal.

This animal ethics clearance letter is valid for only one year (23/11/2024 – 23/11/2025).

Furthermore, any other correspondence and inquiries concerning your research proposal with committee must include the AEEC No., the name of the PI and the proposal title.

Best Regards,

AEEC Chairperson

Name: Nigus Abebe (PhD)

Signature: _____

Date: 23/11/2024

AEEC Secretary

Name: Enquebaher Kassaye (PhD)

Signature: _____

Date: 23/11/2024

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P. O. Box 2084, Mekelle, Tigrai, Ethiopia