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ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

IDENTIFICATION, ANTIMICROBIAL SUSCEPTIBILITY PROFILES AND MOLECULAR DETECTION OF *SALMONELLA* FROM CHICKEN FARMS IN HOLETA, SULULTA AND SEBETA TOWNS, CENTRAL ETHIOPIA

A THESIS SUBMITTED TO COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE OF ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF VETERINARY SCIENCE IN VETERINARY MICROBIOLOGY

BY

EBISA MEZGEBU HAMBISA

ADVISOR

HIKA WAKTOLE (ASSOCIATE PROFESSOR)

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE OF ADDIS ABABA UNIVERSITY

CO-ADVISOR

ABEBE OLANI (ASSOCIATE RESEARCHER)

ANIMAL HEALTH INSTITUTE (AHI)

JUNE, 2022

BISHOFTU, ETHIOPIA

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Department of Microbiology, Immunology and Veterinary Public Health

Title: **Identification, Antimicrobial Susceptibility Profiles and Molecular Detection of *Salmonella* from Chicken Farms in Holeta, Sululta and Sebeta Towns, Central Ethiopia**

Submitted by: Ebisa Mezgebu	________________	_______________
Name of Student	Signature	Date

Approved for submittal to MVSc Research Thesis assessment committee

1. Hika Waktole (Associate Professor)	_________________	_______________
Advisor	Signature	Date
2. Abebe Olani (Associate Researcher)	__________________	_______________
Co-Advisor	Signature	Date
3. Gezahegne Mamo (Professor)	__________________	_______________
Department Chairperson	Signature	Date

Addis Ababa University

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Department of Microbiology, Immunology and Veterinary Public Health

Title: **Identification, Antimicrobial Susceptibility Profiles and Molecular Detection of *Salmonella* from Chicken Farms in Holeta, Sululta and Sebeta Towns, Central Ethiopia**

As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by Ebisa Mezgebu, entitled: **Identification, Antimicrobial Susceptibility Profiles and Molecular Detection of *Salmonella* from Chicken Farms in Holeta, Sululta and Sebeta Towns, Central Ethiopia** and recommended that it be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Veterinary Microbiology.

1. Gebeyehu Goshu (Professor) ________________ ________________

Chairman Signature Date

2. Tadele Tolosa (Professor) ________________ ________________

External examiner Signature Date

3. Gezahegne Mamo (Professor) ________________ ________________

Internal examiner Signature Date

Advisors

1. Hika Waktole (Associate Professor) ________________ ________________

Advisor Signature Date

2. Abebe Olani (Associate Researcher) ________________ ________________

Co-Advisor Signature Date

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my actual work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

Name: Ebisa Mezgebu Signature: ____________

College of Veterinary Medicine and Agriculture, Bishoftu.

Date of submission: ________________________

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LIST OF ABBREVIATIONS

AHI	Animal Health Institute
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Test
BPW	Buffered Peptone Water
BUG	Biolog Universal Growth media
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
IF	Inoculating Fluid
ISO	International Organization for Standardization
Kb	Kilo base
MR	Methyl-Red
OIE	Office of International des Epizooties
PCR	Polymerase Chain Reaction
TSI	Triple Sugar Iron
UK	United Kingdom
USA	United States of America
VP	Voges-Proskauer
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

ABSTRACT

Chickens Salmonellosis is one of the leading causes of heavy losses in chicken industry and has a significant public health impact. In Ethiopian chicken farms, determining the antimicrobial susceptibility test (AST) status of *Salmonella* with respect to its reported serovars was not very prevalent. This study was conducted with the objectives of identification, molecular detection and determination of AST profiles of *Salmonella species* from chickens' farms in central Ethiopia. A cross-sectional study was conducted in selected potential chicken raising areas including Holeta, Sululta and Sebeta from November 2021 to May 2022. A 425 cloacal swabs were sampled by simple random sampling technique, and 18 feed and 18 water samples were collected from 18 farms before providing it for chickens. Out of 461 samples (176, 130 and 155) samples from Holeta, Sululta and Sebeta respectively; 3 (0.65%) *Salmonella* were identified. From these three isolates 2 (1.14%) and 1 (0.65%) were identified from Holeta and Sebeta respectively. However, no sample was found positive from Sululta. Out of the three isolates, 2 (0.47%) and 1(5.56%) *Salmonella* were identified from a total of 425 cloacal and 18 feed samples respectively. Biochemically isolated and Omnilog identified as *Salmonella enterica* Paratyphi B, and *Salmonella* Enterica and *Salmonella* Gallinarum (identified from feed, and the later were from cloacae swabs) samples. The *invA* gene was detected in all of them. Then AST was assessed by 9 antimicrobials of all Oxoid disks; So, *Salmonella* Gallinarum was resistant to streptomycin and tetracycline. Whereas, *Salmonella enterica* Paratyphi B and *Salmonella* Enterica were intermediate to meropenem and streptomycin disks. Only sample type variable was statistically significant ($p < 0.05$). The findings showed that *Salmonella* can be present in chickens and their environments. Even though isolates numbers were low, all of them were resistant and intermediate to some of the antimicrobials; and if it transmitted to other animals and humans with their resistant genes, it can pose a serious risk of transmission of resistant developed genes. This warrants the implementation of strong biosecurity policy, and proper use of antimicrobials by excluding resistance developed antimicrobials from the market. Moreover, awareness should be created to the chicken farm owners on measures to avoid biosecurity and management risk factors of Salmonellosis and the occurrence of antimicrobials resistance in chicken farms.

Key words: *Antimicrobials, Chickens, Detection, Ethiopia, Farms, Identification, Salmonella*

1. INTRODUCTION

Poultry production plays a significant part in the economies and livelihoods of developing countries. The larger proportion of rural poultry in the national flock population of developing countries makes them worth paying attention to improved management and breeding (Farrell, 2013). Chickens are important livestock resources, easily breaking the vicious cycle of poverty and malnutrition in developing countries. The sector serves as a source of cash used to basic assets for children and women. Commercial production hires local people, also it contracts day old chickens to local people to raise it on payment basis (Tadiose *et al.,* 2017).

In Ethiopia, poultry industry is one of the most productive sectors, serving as a key source of protein supplements for the country's rising population. Taking this into account, the Ethiopian Ministry of Agriculture has highlighted poultry production as a major area for addressing food security concerns with the goal of boosting the amount of meat and eggs produced every year by expanding the country's chicken farms (Jenberie *et al.,* 2014).

Most of the chicken diseases were common in Ethiopia. The review by (Asfaw *et al.,* 2019) revealed that 14 infectious and parasitic diseases of poultry were reported in 110 published studies from 2000 to 2017. And 81.82% (90/110) of the studies covered 6 diseases: Newcastle disease, infectious bursal disease, coccidiosis, helminth infestation, ectoparasite infestation, and *Salmonella* infection.

The *Salmonella* genus is one of the most prevalent foodborne pathogens found in food-producing animals that is responsible for zoonotic infections in humans and other animals, including birds. As a result, *Salmonella* infections are a major problem for public health, animals, and the food sector all over the world (Jajere, 2019).

Salmonellosis is the disease caused by *Salmonella serovars* by which few serotypes are host-specific. *Salmonella* Pullorum (*S.* Pullorum) and *S.* Gallinarum are responsible for pullorum disease and fowl typhoid in poultry, respectively, *S.* Typhi for typhoid fever in human beings, *S.* Dublin for disease in bovine, and so on. However, the others are not host-specific and may infect

several animal species including human beings. These serotypes are generally responsible for food-borne diseases and foods of animal origin are the main source. Historically, *S.* Typhimurium is the most common agent of human food-borne disease, although in the past few decades *S.* Enteritidis has become more common. Among all of the *Salmonella* serovars, about eighty are most frequently involved in animal and human salmonellosis. *S.* Typhimurium and *S.* Enteritidis are the most common agents of disease in human beings and animals, but there is also increasing concern about *Salmonella* serotypes such as *S.* Infantis, *S.* Agona, *S.* Hadar, *S.* Heidelberg, and *S.* Virchow (Freitas *et al.,* 2010).

Salmonella Enterica is commonly found in chicken and other food animals, and undercooked poultry items are the most prevalent source of Salmonella infection in humans. The majority of Salmonella isolates from chicken products and farms were found to be resistant to a variety of antimicrobials. The level of public health risk connected with poultry products can be explained using data on farm prevalence and antibiotic susceptibility status of isolates (Eguale, 2018).

One of the most pressing challenges in developing and developed countries is the global increase of food borne diseases due to antibiotic resistant pathogenic bacteria and the spread of antimicrobial resistance (AMR). One related worry for human health is the advent of multi-antimicrobial resistant *Salmonella* strains and the continued spread of its clones. Multidrug resistance in *Salmonella* species and other bacterial pathogens causing gastrointestinal disorders has been identified in a number of countries, it has become a serious public health concern since it has the potential to spread globally (Xu *et al.,* 2020).

Antimicrobial resistance (AMR) refers to bacterial diminished sensitivity to drugs or antibiotics that can cause cell death or growth suppression (Al-sanouri *et al.,* 2008). Antimicrobial sensitivity testing of *Salmonella* isolates to detect susceptibility or resistance to antibiotics can be used to determine this. *Salmonella* resistance is encoded through genes found on chromosomes, extrachromosomal deoxyribonucleic acid (DNA) (plasmids), and transferable genetic materials (transposons, integrons), which can be identified by genetic or molecular methods. Although resistance to antimicrobial compounds can be acquired by mutations in important genetic loci in

the bacterial genome, the majority of resistance to antimicrobial agents is acquired through mobile genetic elements such as plasmids and transposons (Toro *et al.,* 2011).

The identification of unique genetic materials and characterization of mutations in specific genes using polymerase chain reaction (PCR), DNA probes, and other amplification techniques can be used to diagnose resistance genotype (Browne *et al.,* 2020). The use of real-time PCR and molecular fingerprinting of DNA for genotypic characterization of antibiotic-resistant *Salmonella* species has proved successful. Plasmid gene profile analysis is a rapid and very simple way of fingerprinting strains that has been used to analyze the transmission of AMR *Salmonella* in both human and veterinary medicine. Because of its low cost automated procedures, phage typing or alternative genetic techniques, as well as complete DNA sequencing, is increasingly being employed to explore genetic variants in AMR *Salmonella* species (Afolami and Onifade, 2018).

However, the development of resistance in the responsible pathogens has worsened the situation often with very little resources to investigate and provide reliable susceptibility data on which rational treatments can be based as well as means to optimize the use of antimicrobial agents in most of the developing countries (Byarugaba, 2004). *Salmonella* in chickens is one of these resistance developed pathogens having major public health and economic importance throughout the world including developing countries. Determination of antimicrobial susceptibility status of *Salmonella* with respect to its identified serovars was not such common in the chicken farms of our country and there was no previous study carried out in selected study areas in common.

In light of the above background information and existing facts, the present study consisted of the following objectives:

- Identification and molecular detection of *Salmonella* in chicken farms of Holeta, Sebeta and Sululta towns of central Ethiopia
- Determination of antimicrobials susceptibility profiles to the identified *Salmonella* isolates from the selected study areas.

2. LITERATURE REVIEW

2.1 History of Discovery of *Salmonella*

The genus *Salmonella* is a member of the Enterobacteriaceae family named after a famous American veterinary bacteriologist Daniel E. Salmon (1850 - 1914) and become formal in 1934. It was cultured in 1888 by Salmon and Smith from pigs which had died of hog cholera (Rahman *et al.,* 2018).

2.2 Classification, Morphology and Characteristics of *Salmonella*

2.2.1 Classification of Salmonella

The genus *Salmonella* is composed of two species: *S.* Bongori and *S.* Enterica, and it is thought to have split from *Escherichia coli* (*E. coli*) some 100 million years ago. *S.* Bongori is mostly found in reptiles and has only been related with sickness in humans on a few occasions. *S.* Enterica, on the other hand, is composed of thousands of human disease-causing serovars (Gilchrist and Maclennan, 2019). Each of the six subspecies of *S.* Enterica (Enterica, Salamae, Arizonae, Diarizonae, Houtenae, and Indica) has numerous serovars or serotypes. As a result, a serotype is nominated as follows: *S. enterica* subspecies enterica serotype Typhimurium, which may be simplified as *S.* Typhimurium (Freitas *et al.,* 2010).

Salmonella Enterica serovars can be divided into two categories: typhoidal and nontyphoidal strains. The typhoidal serovars, *Salmonella enterica serovar* Typhi (*S.* Typhi) and *S. enterica serovars* Paratyphi A, B, and C (*S.* Paratyphi A, B, and C), are host-adapted, human-restricted organisms, causing a single clinical syndrome, enteric fever, in immunocompetent individuals (Gilchrist and Maclennan, 2019).

Nontyphoidal *Salmonella* is a leading cause of food borne infection worldwide in which its serotypes are usually zoonotic and have a wide range of animal reservoirs (El-ghany, 2006). Some serotypes of *S.* Enterica are considered as emerging zoonotic pathogens, generating outbreaks

worldwide in the human population. It is estimated that *S.* Enteritidis is responsible for about 93.8 million illness and 155,000 deaths worldwide each year, and of these, 80.3 million cases are estimated to be foodborne (Lapierre and Cornejo, 2020). It is a facultative intracellular pathogen that is capable of causing different disease syndromes in a wide range of hosts. *S.* Typhimurium and *S.* Enteritidis are the most often isolated serovars worldwide, resulting in significant economic losses (Ammar *et al.,* 2016).

2.2.2 Morphology and characteristics of Salmonella

Salmonella are Gram-negative, facultative anaerobes that are mostly motile and belong to the Enterobacteriaceae family (Gilchrist and Maclennan, 2019). However, some *S.* Enterica strains such as *Salmonella* Gallinarum and *Salmonella* Pullorum lack flagella and are hence non-motile (Jajere, 2019). The genome of *Salmonella* ranges from 4460 to 4857 Kilo base (kb) (Gut *et al.,* 2018). Biochemically, *Salmonella* is characterized by hydrogen sulfide and catalase production, and lack of oxidase enzyme as well as lactose fermentation property. They are heat-labile and killed at 70°C; however, they can survive in dust and harsh environmental conditions for 2 years and more. *Salmonella* isolates are often classified by their serotype. *Salmonella* serotyping is based on identifying somatic antigen (O) and flagellar antigen (H) found in the bacteria's flagella (El-Saadony *et al.,* 2022).

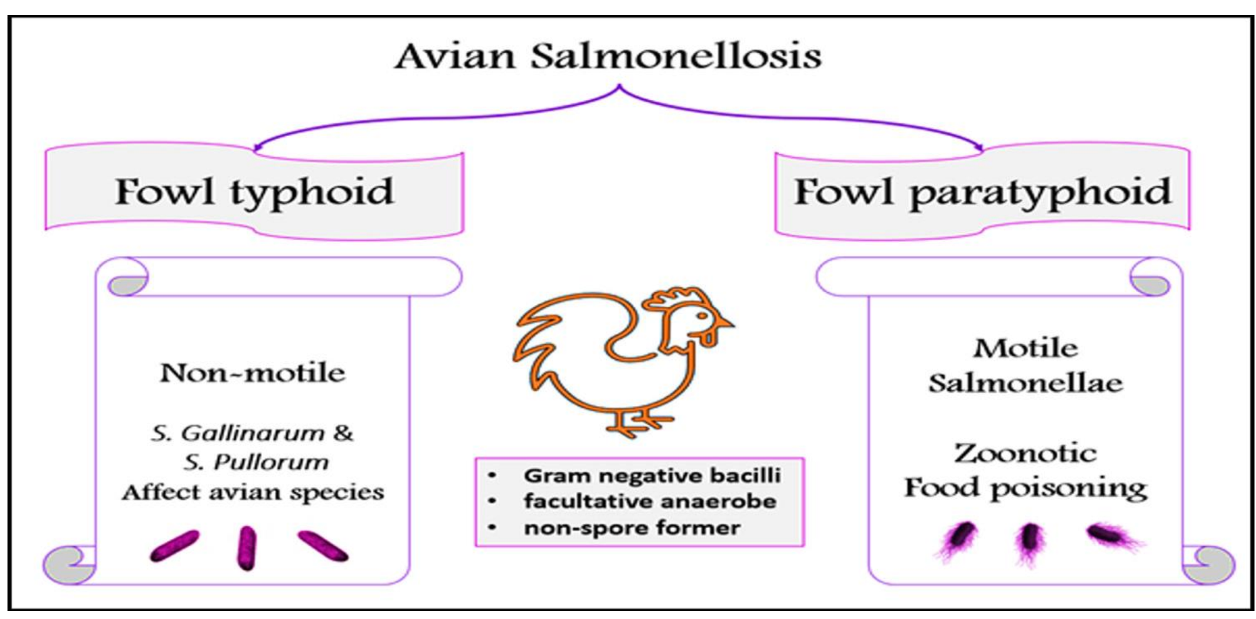

Figure 1: Avian Salmonellosis and its bacteriological characters

Source: (El-Saadony *et al.,* 2022).

2.3 Sources and Reservoirs of Chicken Salmonellosis Infection

Live chickens as well as their products are incriminated as major sources of human food borne Salmonellosis. PCR was used for detection of the similarity between different *Salmonella* serovars from different sources and the results yielded similar amplified DNA bands in *S.* Enteritidis and *S.* Typhimurium of the same chickens and human origin. Similarly, *S.* Enteritidis was identified from chicken's meat and patient with food poisoning signs. Poultry products are also considered as the most important sources of *Salmonella* transmission to human (Tarabees *et al.,* 2017). Egg shell contamination among Egyptian layers flocks plays an important role in transmission of disease to human (El-Prince *et al.,* 2019).

2.4 Modes of Chickens *Salmonella* Transmission

The spread of *Salmonella* is very normal and persistent in dry environments, but it can last for up to several months in water. In animals that are sometimes considered "carriers," *Salmonella* can cause clinical or sub-clinical infection in asymptomatic animals. Its mode of transmission can be

horizontal or vertical, and its spread from the parent to the infant is vertical transmission. In *Salmonella* infection in poultry which is caused by serovar Enteritidis with a particular preference for the chicken reproductive system, vertical transmission is of great concern. In this case, it happens through transovarian infection when the mother bird has a systemic infection that results in ovary infection and egg production in the oviduct. Bacteria migrating from cloaca into reproductive organs contribute to serovar Enteritidis gaining access to eggs. Horizontal transmission takes place through aerogens and/or faeco-oral transmission. *Salmonella* is clearly transmitted horizontally by fomite, contaminated drinking water, contaminated feed, dirty cages, no signs of infected animals and faeces of infected livestock (Wibisono *et al.,* 2020).

Some of the investigations of chicken Salmonellosis outbreaks apart from those related to contact with live poultry, there are also indirect contact of infected or contaminated food vendors (Loharikar *et al.,* 2013). Another example of an epidemic caused by indirect contact with live poultry is foodborne disease which originates from workers who are infected by direct contact with chickens from the farm (Hedican *et al.,* 2010). Animals are the main reservoir for transmission of non-typhoid *Salmonella* (NTS) infection. The incidence of NTS around the world is high, and can be found in the environment. It is this diversity of reservoirs that creates significant difficulties for public health authorities in the control of infection (Dione *et al.,* 2011).

Significant reservoirs for *Salmonella* are man, farm animals, pigeons, waterfowl and wild birds. Rodents, pets and insects are also potential reservoirs and transmit the infection from bird to bird and between houses. The organisms are often localized in the gut of these carriers, which shed *Salmonella* intermittently in the faeces thus contaminating the poultry environment. Probably one of the most common factors for horizontal spread of the organisms is feed. Nearly every ingredient ever used in the manufacture of poultry feedstuffs has been shown at one time or another to contain *Salmonella* (Hafez, 2016).

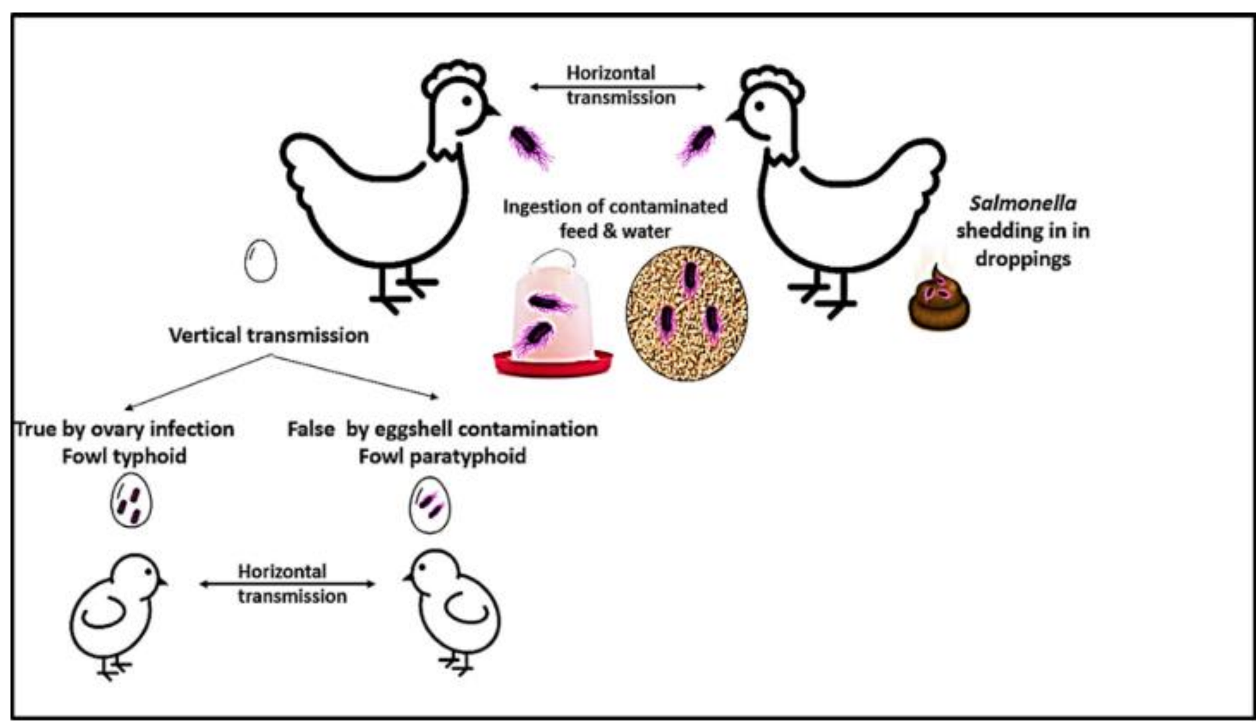

Figure 2: Modes of transmission of Chicken Salmonellosis

Source: (El-Saadony *et al.,* 2022)

2.5 Diagnosis of *Salmonella* in Chickens

Clinical signs and lesions have little value in diagnosis, because in many cases the infection does not accompanied with clinical disease or lesions. Accurate diagnosis must be substantiated by isolation, identification and/or detection of antibodies using serological examination (Hafez, 2016).

2.5.1 Isolation and identification

Most of the time, an organism can be detected in hatching eggs, dead-in-shell embryos, heart blood, liver, spleen, kidney, crop, intestinal content, unabsorbed yolk, faeces and environmental samples such as drag swabs, floor litter, nest litter and dust. The diagnosis of *Salmonella* can be carried out by procedures described on ISO 6579-1:2017(E) including pre-enrichment in non-

selective liquid medium, enrichment in/on selective media, plating out on selective solid media and confirmation by means of appropriate biochemical and serological tests (ISO, 2017).

2.5.2 Identification of Salmonella by Omnilog

The Omnilog identification System includes everything you need to incubate microbes and use all the features of Omnilog data collection software. Omnilog ID is a dedicated system for bacterial identification and has the following components including: dedicated Windows-based computer with preinstalled Omnilog data collection software, Omnilog Incubator/Reader, Printer, turbidimeter, and electronic multichannel repeating pipette. Biolog's technology uses each microbe's ability to use particular carbon sources or chemical sensitivity assays to produce a unique pattern or "Phenotypic Fingerprint" for that microbe. The Biolog GEN-III MicroPlate provides 94 phenotypic tests: 71 carbon source utilization assays and 23 chemical sensitivity assays. The test panel provides a "Phenotypic Fingerprint" of the microorganism that can then be used to identify it at the species level (BIOLOG, 2010).

2.5.3 Serological tests

In chickens, a number of serological tests have been developed for the diagnosis of *Salmonella* infections. The whole blood test, which uses a stained antigen, and the serum agglutination test have been used successfully for over 50 years for the identification of flocks infected with *S*. Gallinarum (biovars Gallinarum and Pullorum). *S*. Enteritidis possesses the same group D somatic antigen as *S*. Gallinarum and is thought to originate from a common ancestor, the whole blood test and related tests can be used for the diagnosis of *S*. Enteritidis infection. Other tests, such as the ELISA have been developed for the diagnosis of *S*. Enteritidis and *S*. Typhimurium infections in poultry (OIE, 2022).

2.6 Antimicrobial Resistance in Chicken Salmonellosis

2.6.1 Mechanisms of antimicrobials resistance

Different mechanisms of AMR development have been reported and these mechanisms, leads to the emergence of multidrug resistance. These can be divided into two broad mechanisms: biochemical and genetics mechanisms, and these are enzymatic inactivation, drug permeability reduction, active efflux pumps, biofilm formation, antimicrobial targets alteration, mutation, vertical and horizontal genes transfer (Akinyemi and Ajoseh, 2017).

2.6.2 Diagnosis of antimicrobial resistance

Diagnosis of AMR can be carried out by different methods (Kaprou *et al.,* 2021) and these involve disk diffusion, agar dilution, gradient test and broth microdilution (Otiso *et al.,* 2019), detection of Antimicrobial Resistant Genes (ARGs) by PCR (El Seedy *et al.,* 2017), sequencing and other techniques (Teng *et al.,* 2017).

2.6.3 Causing factors for antimicrobial resistance development

The factors identified as a driving for AMR development are: misuse of antimicrobials, contamination of the environment, mass drug administration and incorrect dosing of antibiotics (Castro-Sanchez *et al.,* 2016) and it is mainly driven by inappropriate use. In many countries, antibiotics can be bought without prescription or do not have underlying standard treatment guidelines. These factors increase antibiotic resistance because of a lack of knowledge of proper antibiotic use (WHO *et al.,* 2016).

The utilization of antimicrobials for growth promotion of animals, for prophylaxis and for the treatment of the diseases caused by bacterial pathogens can lead to development of AMR in pathogens. In different studies, both pig and chicken meats have been documented as the reservoir for drug-resistant *Salmonella species*. This transmission of drug resistance through the food chain is considered as a major public health concern (Xu *et al.,* 2020).

2.7 The Status of Chicken Salmonellosis in Ethiopia

In Ethiopia, many studies were conducted on the Chicken Salmonellosis prevalence and some of the authors indicated as shown in (table 1) below:

Table 1: Prevalence of Chicken Salmonellosis in Ethiopia in different geographical areas

Study area	Tested Chicken	Prevalence (%)	Type of test	Authors and years	Sample type
Hawassa	380	0.8	Biochemical and Analytical Profile Index (API) 20E	(Aragaw *et al.,* 2010)	Cloacal swab
Kombolcha	400	11.5	Biochemical	(Assefa *et al.,* 2011)	Egg
Debre Zeit	150	9.33	Serum agglutination and Biochemical	(Endris *et al.,* 2013)	Serum, cloacal swab
Jimma town	384	41.9	Biochemical	(Kindu and Addis, 2014)	Faeces
Haramaya	300	2.7	Biochemical	(Kemal *et al.,* 2016)	Eggs
Hawassa and Bonga	270	16.67	Biochemical	(Abdi *et al.,* 2017)	Cloacal swab, Hand swab and bedding swab
Central Ethiopia	549	14.6	Biochemical, Serum agglutination and PCR	(Eguale, 2018)	Faeces
Debre Zeit and Modjo	384	14.6	Biochemical	(Ali *et al.,* 2020)	Caecal contents
Kafa zone	302	9.27	Biochemical	(Abda *et al.,* 2021)	Cloacal swab
Bishoftu and Modjo	539	24.3	Biochemical	(Belachew *et al.,* 2021)	Feed, water, and water- and feed-trough samples, Caecal content

2.8 Antimicrobial Susceptibility Profiles of Chicken Salmonellosis Status in Ethiopia

Contaminated food with drug-resistant microorganisms is the most vital hazard as antimicrobial resistance can switch to other bacteria. The emergence and spread of antimicrobial-resistant *Salmonella* originating from food animals have grown to be a serious health hazard worldwide, especially in growing countries (Asefa and Tsegaye, 2022).

The study carried out by (Dagnew *et al.,* 2020) by identification of *Salmonella* isolates and subjecting them to 15 antimicrobial disks and investigated that one or more isolates demonstrated resistance to streptomycin, tetracycline, nitrofurantoin, sulfisoxazole, neomycin, and kanamycin. The percentage of isolates resistant to streptomycin, tetracycline, nitrofurantoin, sulfisoxazole, neomycin, and kanamycin accounted for 20 (80%), 19 (76%), 11 (44%), 8 (32%), 6 (24%), and 3 (12%), respectively. Thirteen out of 14 (92.9%) and 14 (100%) of *S*. Haifa isolates were resistant to streptomycin and tetracycline, respectively. The study conducted by (Mohammed and Dubie, 2022) on poultry farms in Addis Ababa and tested for 23 identified *Salmonella* isolates by using 10 antimicrobial disks; 95.65% of them were resistant to one or more antimicrobials. Multiple drug resistant were observed for 69.56% of *Salmonella* isolates. The highest resistance 73.9% was observed in kanamycin followed by tetracycline (65.2%) and streptomycin (56.3%). Gentamycin was the most effective antibiotic (95.7%) sensitivity followed by ciprofloxacin (78.3%) sensitivity and ampicillin (69.6%) sensitivity.

The study investigated by (Kemal *et al.,* 2016) by performing on 8 isolates of *Salmonella* were tested against 12 antimicrobials. Of the antimicrobials tested, *Salmonella* isolates were all resistant to erythromycin and clindamycin. Isolates were sensitive to ciprofloxacin (100%) and chloramphenicol (87.5%). All isolates were resistant to multiple antibiotics. In a similar way, (Woyessa *et al.,* 2021) also conducted AST on 37 *Salmonella* isolates against 15 antimicrobials using disc diffusion method. Majority of the isolates (24/37) were resistant or intermediately resistant to at least one antimicrobial. The prevalence of resistance was high to chloramphenicol (62.2%), tetracycline (59.5%), ampicillin (54.1%) and streptomycin (51.4%). More than half of the isolates (56.8%) were multidrug resistant.

The other study investigated on 28 *Salmonella* isolates against 14 antimicrobials showed, all isolates were 100% resistant to Oxytetracycline and Ampicillin. Among 28 isolates, 26 (92.85%) of them were showed multidrug resistance while 2 (7.14%) of them showed extensively drug resistance. Half of multidrug-resistant isolates were resistant to 5–6 antimicrobials, while 7.14% of isolates showed resistance to 7 antimicrobials (Abda *et al.,* 2021).

3. MATERIALS AND METHODS

3.1 Study Area

The study was conducted in selected parts of central Ethiopia which are found in the Oromia Special Zone Surrounding Finfinne including Holeta, Sebeta and Sululta towns. The study was performed from November 2021 to May 2022 in chicken farms of different production systems (Figure 3). Holeta is located at 40 Km to the west of Finfinne. It has a latitude and longitude of 9°3′N 38°30′E / 9.050°N 38.500°E and an altitude of 2398.98 meters above sea level with an average temperature of 15.76 ℃ and 900 to 1100 mm annual rainfall. Sebeta is located at 22 Km distance to the South-west of Finfinne. This town has a latitude and longitude of 8°54′N 38°37′E / 8.911°N 38.621°E and an elevation of 2,356 meters above sea level. The average temperature is 17.4 ℃ and the town receives an annual rainfall of 1650 mm, the monthly precipitation is being 150 mm are mostly wet and below 30 mm mostly dry. Sululta town is located at 32 Kms to the north of Finfinne, and has a latitude and longitude of 9°12′N 38°72′E / 9°7′N 38°43′E Finfinne, and it has an elevation of 2,785 metres with an average annual temperature 15.36 ℃ with the mean minimum of 6.2 ℃ in December and maximum 22.9 ℃ in February and May. In all of the three study areas, small scale and medium scale commercial chicken production systems were practiced.

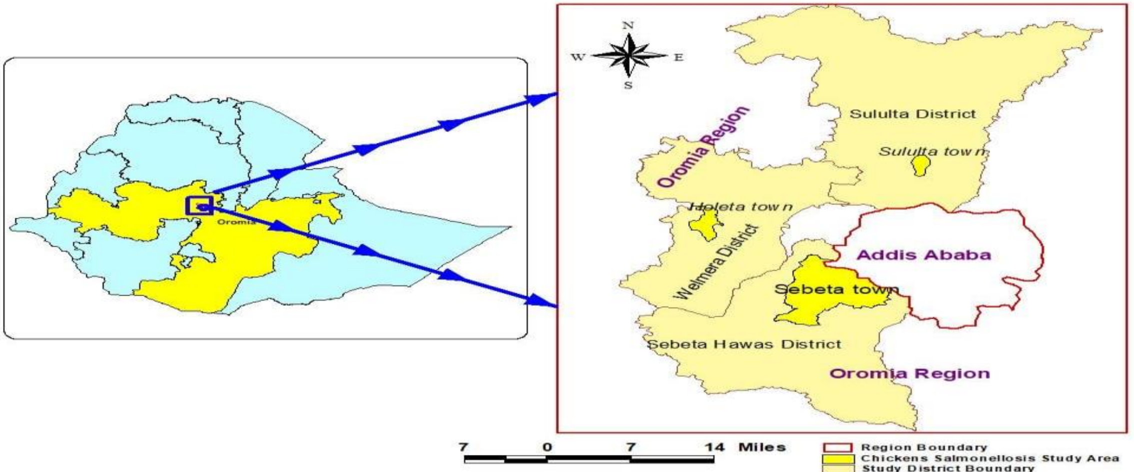

Figure 3: Map of the study area

3.2 Study Population

The study animals were chickens raised under small scale (50 – 1000 chickens) and medium scale (1001-10, 000 chickens) commercial production systems (Amare *et al.,* 2021). Young and adult chickens were included in the study and their ages were grouped in to young (≤ 24 weeks) and adult (> 24 weeks) according to (Sarba *et al.,* 2020). Broilers and layers were involved in the study.

3.3 Study Design and Sample Type

The central part of Ethiopia was selected purposively for being the major chicken raising area of the country where a number of farms of various scale are concentrated. Then, from central part of Ethiopia, the three study sites were randomly selected involving Holeta, Sululta, and Sebeta towns and its surroundings. The present study employed a cross sectional study design to address the research objectives in the three selected study areas. Lists of farms were obtained from Districts' Veterinary Offices. The samples that were collected included cloacae swabs which was sampled by simple random sampling technique from apparently healthy chickens, and feed and water samples from each selected farms before providing it for chickens.

3.4 Sample Size Determination, Collection and Sampling Method

3.4.1 Sample size determination

The sample size was determined by using (Thrusfield, 2007) formula with an expected prevalence of 50% and with 0.05 precision, and the sample size at a 95% confidence interval was calculated by the formula:

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

Where:

n = required sample size

P_{exp} = expected prevalence

d = desired absolute precision

Therefore, sample size at expected prevalence of 50% was;

$$n = \frac{1.96^2 (0.5)(1 - 0.5)}{0.05^2}$$

$$= \frac{3.84(0.5)(0.5)}{0.0025}$$

= 384 samples

However, to increase the precision, 20% of a calculated sample size was added and, accordingly, 20% of the calculated sample size was about 77 samples, and therefore, a total of 461 samples were tested for the study.

3.4.2 Sample collection and sampling method

For the collection of cloacal swab, both wings of the chickens were held with one hand, keeping the heads down to expose their caudal parts. The cloacal surface was disinfected by 70% alcohol for 2 minutes before collection of sample. A moistened sterile cotton swab with buffered peptone water was inserted and rolled inside in the cloacae many times and transferred as soon as in to the universal bottles with 9 ml of buffered peptone water (BPW, Oxoid CM0509, UK) and kept in an icebox with icepack. Feed and water samples were collected before being provided to the chickens. A sterile plastic bags and bottles were used for the collection of 25 grams of feed and 5 ml of water samples respectively (Belachew *et al*., 2021). All the collected samples were labelled and kept in an icebox with an icepack, and transported to Animal Health Institute (AHI) according to (OIE, 2013) procedures for the identification**,** antimicrobial susceptibility determination and molecular detection of *Salmonella* from chickens . Additionally, information including farm type, flock size, age, type of chickens and other related information were recorded from each farms included in the study. And the (table 2) below indicated the type and number of samples collected with their respective areas.

Table 2: Type and number of samples collected with their respective study areas

Study area	Sample type			
	Cloaca swab	Feed	Water	Total
Holeta	162	7	7	176
Sebeta	143	6	6	155
Sululta	120	5	5	130
Total	425	18	18	461

3.5 Isolation of *Salmonella*

The isolation of *Salmonella* was performed according to the ISO 6579-1:2017(E) (ISO, 2017) protocol and required the stages as described below:

3.5.1 Pre-enrichment in non-selective medium and enrichment in/on selective media

The swabs samples were pre-enriched in 9 ml of BPW (Oxoid CM0509, UK) and incubated for 24 hours at 37 °C. Following incubation in BPW, 0.1 ml of the pre-enrichment broths was aseptically transferred to 10 ml of Rappaport-Vassiliadis Soya (RVS) broth (Himedia M1491-500G, India), mixed and incubated for 24 hours at 41.5 °C in the Class II biosafety cabinet.

3.5.2 Plating out on selective solid media

After 1 day of RVS broth culture incubation at 41.5 °C, a loopful of a culture was streaked onto xylose lysine deoxycholate (XLD) agar (Himedia M031-500G, India) and incubated at 37 °C for 48 hours. The XLD agar plates were examined for the presence of *Salmonella* colonies. Red colonies with black centers on XLD agar plates were considered as presumptive suspected for *Salmonella*, and further purified by culturing on nutrient agar (Criterion, C6461,USA) (WHO, 2010).

3.6 Identification and Detection of *Salmonella* and Its Antimicrobial Susceptibility Test

3.6.1 Biochemical tests identification

Colonies of presumptive *Salmonella* were sub-cultured from XLD agar to nutrient agar (Criterion, C6461, USA) and their identity were confirmed by means of different biochemical tests. Accordingly, presumptive *Salmonella* colonies were picked up from the nutrient agar and inoculated into urea agar, triple sugar iron (TSI) agar, indole, Simmons citrate agar, MR-VP broth (Oxoid, CM 0043, UK) and Lysine broth, and were incubated for 24 hours at 37 °C (WHO, 2010).

Colonies producing an alkaline slant with acid (yellow color) butt on TSI with hydrogen sulfide production, negative for urea hydrolysis (red color), positive for lysine (purple color), negative for indole test (yellow-brown ring), negative for Voges-Proskauer, positive for MR and positive for citrate utilization were considered as *Salmonella*-positive (Woyessa *et al*., 2021).

3.6.2 Omnilog identification of Salmonella

To identify *Salmonella*, the isolate to be identified was grown on Biolog Universal Growth (BUG) agar medium and then a single colony was suspended in a special "gelling" inoculating fluid (IF-A) using inoculazerat the recommended cell density. Then, 100 μL of the cell suspension was inoculated into a well of the GEN-III MicroPlate, and the MicroPlate was incubated to allow the phenotypic fingerprint to form. After incubation for 22 hours at 33 ºC the phenotypic fingerprint pattern was read by a combination of the Biolog Microstation reader. The fingerprint data was imported into Omnilog Data Collection software, which searched an extensive database and made an identification call in seconds. The identification process of *Salmonella* involved four main steps. These steps were isolation of a pure culture on Biolog media, preparation of inoculum, inoculation of MicroPlates and load into the reader, and obtaining of ID results from the printer (BIOLOG, 2010).

3.6.3 Molecular detection of Salmonella

DNA extraction

The thermal block was heated to 95 °C. 100 µl of nuclease free water was pipetted in a micro centrifuge tube and a single colony was touched with a loop and re-suspended in nuclease free water by swirling the loop in a tube 10 times and vortex the tube for 30 seconds. Then a tube was placed in a thermal block and incubated for 10 minutes and after incubation the tube was cooled down for 2 minutes at ambient temperature. Next, it was centrifuged at the highest speed 14,000 rpm for 5 minutes and 50 µl of the supernatant was transferred to a fresh 1.5ml micro centrifuge tube and stored at −20°C (Bio-rad, USA).

Real-time Polymerase Chain Reaction detection of *Salmonella*

Extracted DNA was detected for *Salmonella* by amplifying the *invA* gene, and using specific primers as shown in table 1. A known *Salmonella* isolate in the laboratory was used as a positive control and a nuclease free water was used as a negative control. The PCR reaction volume was 25µl which consisted of 12.5µl iQ Multiplex Powermix 2x, 0.5 µl of each forward and reverse *invA* primer mix, 8.5 µl of nuclease free water, 0.5 µl of *invA* probe and 2.5 µl of DNA template (Bio-rad, USA). The PCR conditions for amplification were 95 °C for 10 minutes, was followed by 45 cycles of 15 seconds at 95 °C and 50 seconds at 60 °C.

Table 3: Primers and probe sequences

Salmonella gene	Name	Sequence	Amplicon Size (bp)	Reference
invA	*invA*-F	5’-CGTGTTTCCGTGCGTAATA-3’	138	(Ibrahim *et al.,* 2014)
	invA-R	5’-GCCATTGGCGAATTTATG-3’		
	invA-Pr	5’-FAM-ATTATGGAAGCGCTCGCATT-BHQ1-3’		

3.6.4 Antimicrobial susceptibility test (AST)

After biochemical test, isolation and identification of pure isolates of *Salmonella* with Omnilog, antimicrobial susceptibility testing against selected drugs was determined using the Kirby–Bauer disk diffusion method and Clinical and Laboratory Standards Institute (CLSI) method (CLSI, 2020). Refreshed pure isolated colonies from the nutrient agar plates were transferred into tubes containing 5 ml of 0.85% of sterilized saline water. Then, it was measured by McFarland Densitometer until it achieved 0.5 McFarland turbidity standards. A sterile cotton swab was used to swab the inoculum uniformly over the surface of Mueller Hinton Agar (Criterion, C6421, USA) plate. The Plates were held at room temperature for 30 minutes to dry.

The antimicrobial disks used in the study were nine and include: ampicillin (AMP 10 µg), gentamicin (CN 10 µg), ceftriaxone (CRO 30 µg), streptomycin (S 10 µg), tetracycline (TE 30 µg), amoxycillin-clavulanic acid (AMC 30 µg), sulphamethoxazole-trimethoprim (SXT 25 µg), meropenem (MEM 10 µg) and trimethoprim (W 5 µg), and all these antimicrobials were of oxoid, UK.

The pre-determined antimicrobial disks were then dispensed into the bacterial lawn by Oxoid antimicrobial susceptibility disk dispenser. The plates were incubated at 37 °C for 18 hours. Zones of inhibition around the disks were measured to the nearest millimeter using a caliper, the size of the zone of inhibition were compared with CLSI standard and classified as sensitive (S), intermediate (I) or resistant (R) to the antimicrobial disks used as shown in the table 2, and a known isolate of *Salmonella* in the laboratory was used as a control reference strain.

Table 4: Interpretive categories and zone diameter break-points of antimicrobial disks

Antimicrobial agents	Disk content	Interpretive categories and zone diameter break-points (mm)		
		Susceptible	Intermediate	Resistant
Ampicillin (AMP)	10 µg	≥ 17	14-16	≤ 13
Amoxycillin-clavulanic acid (AMC)	30 µg	≥ 18	14-17	≤ 13
Ceftriaxone (CRO)	30 µg	≥ 23	20-22	≤ 19
Meropenem (MEM)	10 µg	≥ 23	20-22	≤ 19
Gentamicin (CN)	10 µg	≥ 15	13-14	≤ 12
Streptomycin (S)	10 µg	≥ 15	12-14	≤ 11
Tetracycline (TE)	30 µg	≥ 15	12-14	≤ 11
Sulphamethoxazole-trimethoprim (SXT)	25 µg	≥ 16	11-15	≤ 10
Trimethoprim (W)	5 µg	≥ 16	11-15	≤ 10

Source: (CLSI, 2020).

3.7 Questionnaire Survey

Questionnaire survey was administered to chicken farm owners to assess and associate the general management practices of the chicken farms with the occurrence of *Salmonella*. Additionally, the questionnaire survey considered and assessed the flock size, purpose of breeding (layer or broiler), age, presence or absence of Personal protective equipment (PPE) including boats, overalls and others, footbath, entrance of other animals in to the farm, using of antimicrobials, and other practices were recorded and analyzed.

3.8 Data Management and Analysis

The data collected during the study that involved questionnaire survey data and other laboratory investigations were coded and stored into Microsoft Excel spread sheet 2010 and the prevalence of *Salmonella* was calculated as a percentage of Omnilog identified *Salmonella* positive samples

among the total number of samples examined. A Chi-square test and others was analyzed by STATA version 13 to observe the association between *Salmonella* isolates and risk factors. The significance level was set at 0.05 alpha value and 95% confidence level.

3.9 Ethical Clearance

Ethical clearance was offered by Animal Research Ethics Review Committee of the Addis Ababa University College of Veterinary Medicine and Agriculture Bishoftu, Ethiopia, and it's Ref No: was VM/ERC/09/04/13/2021.

4. RESULTS

4.1 Prevalence of *Salmonella*

Overall, 3 (0.65%) *Salmonella* were identified from the total 461 samples examined. From these 3 isolates of *Salmonella,* 2 (1.14%) were identified in 176 samples originated from Holeta and 1 (0.65%) were identified in 155 samples tested from Sebeta. However, no sample was found positive from 130 samples collected from Sululta. Out of these 3 isolates, as presented in table 3, 2 (0.47%) *Salmonella* were identified from a total of 425 cloacal samples tested and 1 (5.56%) *Salmonella* isolate was identified from 18 feed samples of chicken. However, there were no isolates of *Salmonella* identified in water samples collected from chicken farms.

4.2 Identification and Detection of *Salmonella*

From a total of 461 samples pre-enriched in BPW broth (Oxoid CM0509, UK), enriched in RVS broth (Himedia M1491-500G, India), and plated out on XLD agar (Himedia M031-500G, India), three samples showed red colonies with black centers on XLD agar plate (Figure 4) and were presumptive for *Salmonella* and confirmed by using different biochemical tests.

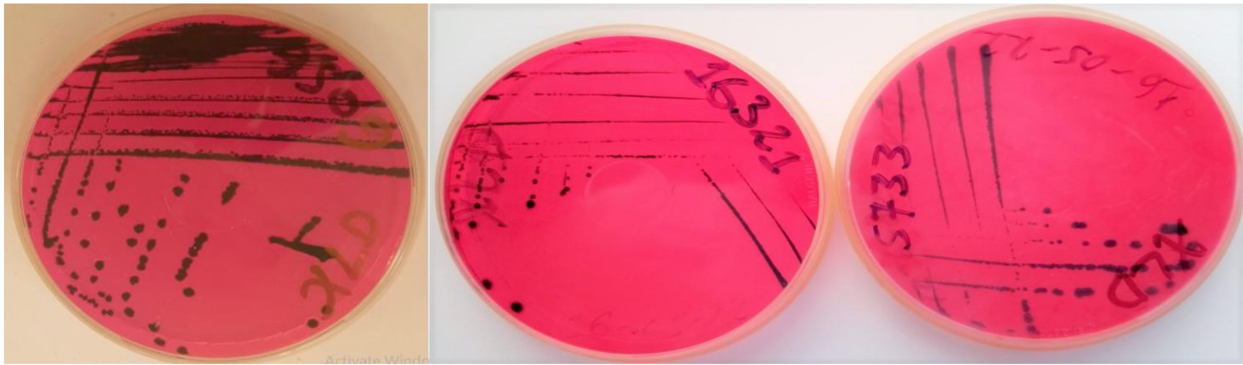

Figure 4: Red colonies with black centers of presumptive *Salmonella* positive on XLD agar

4.2.1 Biochemical test identification

Out of the 461 collected samples, the suspected samples were subcultured on nutrient agar and confirmed by biochemical tests for *Salmonella* identification, and additionally, motility test was performed, and one of the isolates was non motile, while the other left were motile and the results of the 3 isolates for each tests on respective media were presented on (Figure 9) below.

Table 5: Typical reactions of *Salmonella* to different biochemical tests

Samples	Biochemical test results							
	Urease	Citrate	TSI	Lysine decarboxylase	Indole	VP test	Motility	MR
5500	-	+	Acid butt, alkaline slant and H_2S +	+	-	-	-	+
5733	-	+	Acid butt, alkaline slant and H_2S +	+	-	-	+	+
16321	-	+	Acid butt, alkaline slant and H_2S +	+	-	-	+	+

4.2.2 Omnilog identification of Salmonella

After the isolation of 3 *Salmonella* by using of biochemical tests, these isolates were subjected to the Omnilog identification. Accordingly, all of the 3 samples were *Salmonella* positive, and one of them was identified as *Salmonella enterica* ST Paratyphi B which was identified from chicken feed, however, the left two were identified as *Salmonella* Enterica and *Salmonella enterica* ST Gallinarum respectively and they were identified from the cloacae swab samples.

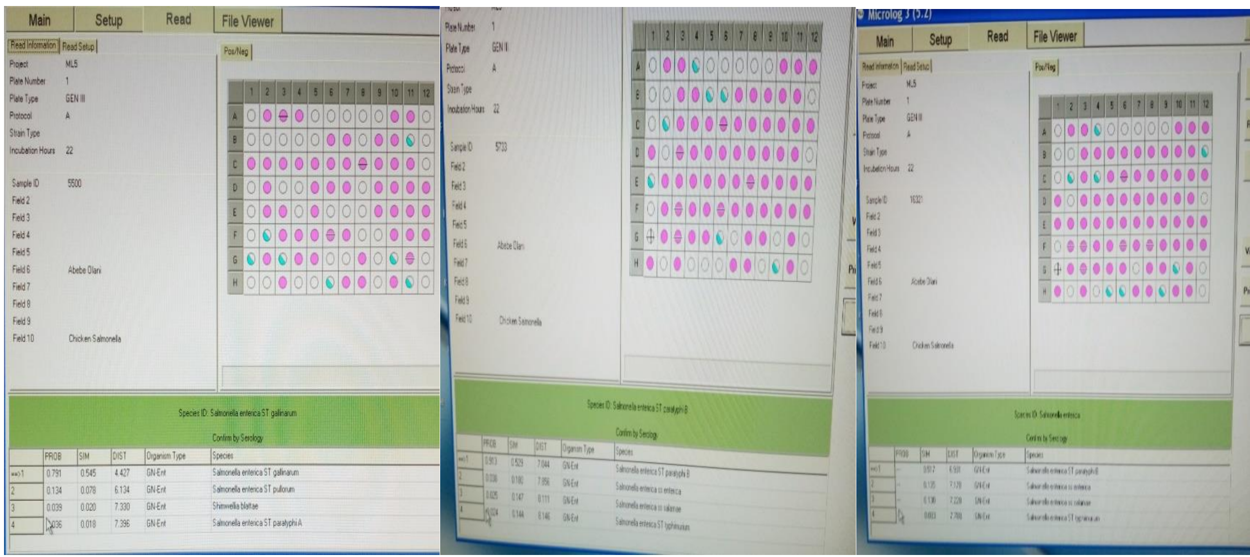

Figure 5: Omnilog identified *Salmonella species*

4.2.3 Molecular detection of Salmonella

After DNA was extracted from the three *Salmonella* isolates, it was detected by quantitative real time polymerase chain reaction by amplifying the *invA* gene, using specific primers of *invA* F: 5' CGTGTTTCCGTGCGTAATA 3', *invA* R: 5' GCCATTGGCGAATTTATG 3'and specific probe: *invA*-Pr: 5'-FAM-ATTATGGAAGCGCTCGCATT-BHQ1-3. All of the detected samples showed that they were positive for *Salmonella* (figure 6).

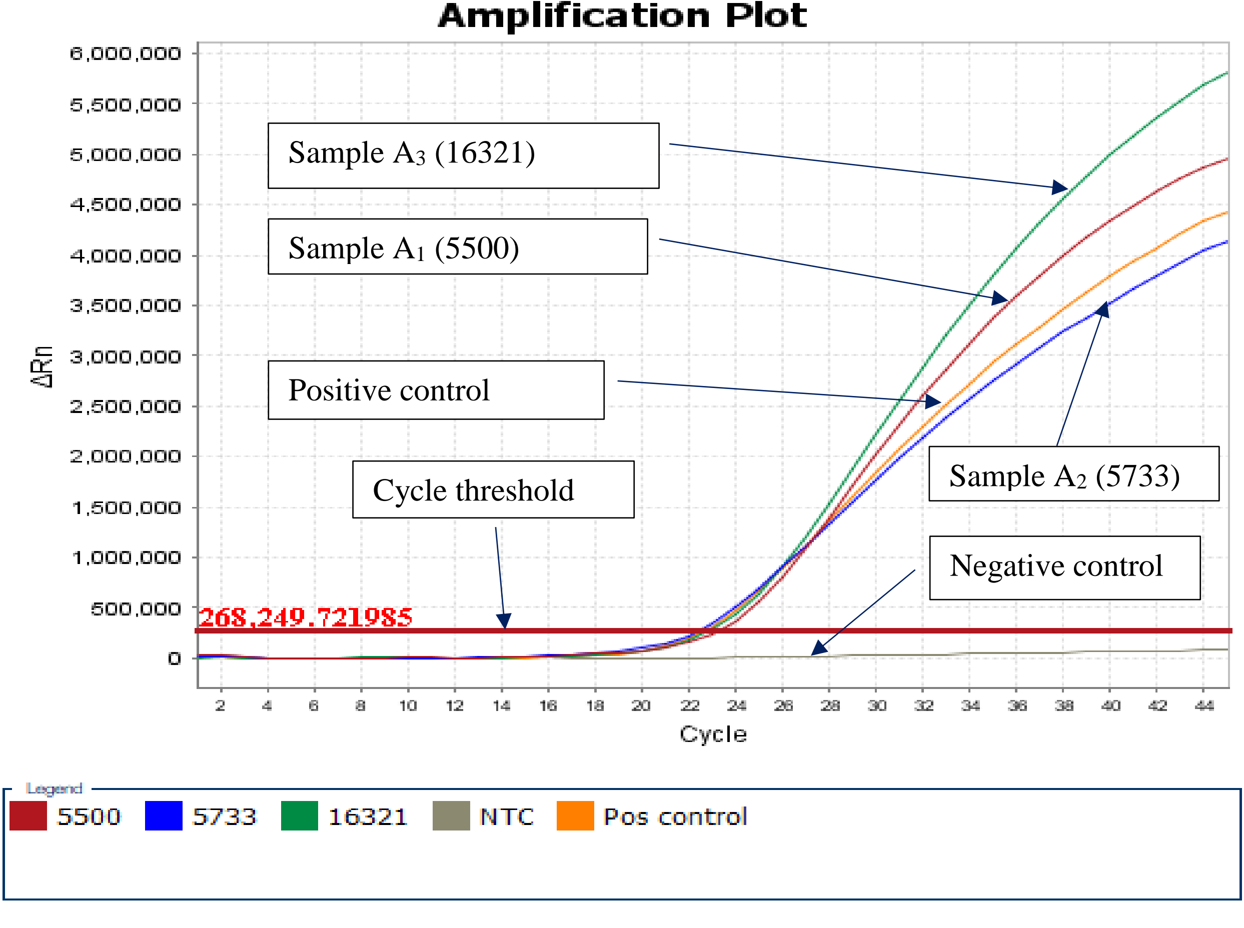

Figure 6: Amplification plot of *Salmonella* positive samples and controls

4.3 Antimicrobial Susceptibility Profiles

All of the 3 isolates of identified *Salmonella* were tested for their antimicrobial susceptibility. From all of the 9 antimicrobial disks applied to assess the antimicrobial susceptibility, *Salmonella enterica* ST Gallinarum was resistant to 2 drugs including streptomycin and tetracycline. In another way, both *Salmonella enterica* ST Paratyphi B and *Salmonella* Enterica were intermediate to both of the meropenem and streptomycin disks. However, all the isolates were susceptible to the rest of antimicrobials as shown in the (table 7) below.

Table 6: Antimicrobial Sensitivity Test Readings

Samples	Antimicrobial Sensitivity Readings																	
	AMC (30µg)		AMP (10µg)		CN (10µg)		CRO (30µg)		MEM (10µg)		S (10µg)		SXT (25µg)		TE (30µg)		W (5µg)	
5500	31	S	19	S	20	S	31	S	26	S	7	R	25	S	6	R	26	S
5733	25	S	20	S	23	S	29	S	22	I	14	I	28	S	22	S	27	S
16321	25	S	20	S	22	S	28	S	21	I	14	I	26	S	21	S	27	S

S: Susceptible, I: Intermediate, R: Resistant

Table 7: Antimicrobial susceptibility test status

Antimicrobial agents	Antimicrobial Sensitivity Status		
	Susceptible (%)	Intermediate (%)	Resistance (%)
Ampicillin (AMP)	3 (100.00%)	0 (0.00%)	0 (0.00%)
Amoxycillin-clavulanic acid (AMC)	3 (100.00%)	0 (0.00%)	0 (0.00%)
Ceftriaxone (CRO)	3 (100.00%)	0 (0.00%)	0 (0.00%)
Meropenem (MEM)	1 (33.33%)	2 (66.67%)	0 (0.00%)
Gentamicin (CN)	3 (100.00%)	0 (0.00%)	0 (0.00%)
Streptomycin (S)	0 (0.00%)	2 (66.67%)	1 (33.33%)
Tetracycline (TE)	2 (66.67%)	0 (0.00%)	1 (33.33%)
Sulphamethoxazole-trimethoprim (SXT)	3 (100.00%)	0 (0.00%)	0 (0.00%)
Trimethoprim (W)	3 (100.00%)	0 (0.00%)	0 (0.00%)

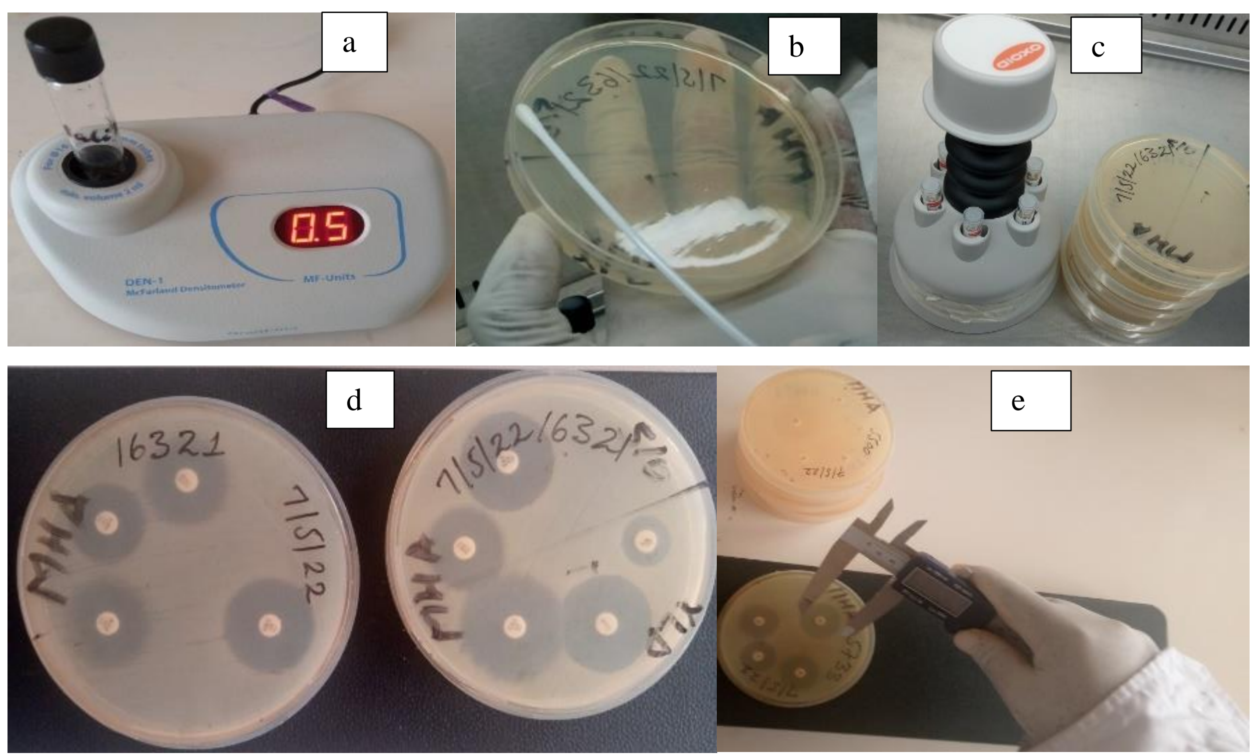

Figure 7: Some of the activities carried out during AST work

a) McFarland densitometer b) Swabbing of bacterial culture on MHA
c) Disk dispenser with antimicrobial disks
d) Zones of antimicrobial susceptibility on MHA
e) Measuring of zones of inhibition around the disks using a caliper

4.4 Results of the Questionnaire Survey

The results of responses from Chicken farm owners and data collected from each of the farms were analyzed and from all the variables only sample type was statistically significant ($p < 0.05$), however other variables were not statistically significant ($p > 0.05$) and presented as in the (table 8) below:

Table 8: Results of the data obtained from the questionnaire survey

Variables	Categories	Tested samples	Positive samples	Chi-square	P-Value
Area	Holeta	176	2 (1.14%)	1.4935	0.474
	Sululta	130	0 (0.00%)		
	Sebeta	155	1 (0.65%)		
Sample type	Cloacal swab	425	2 (0.47%)	7.0291	0.030 *
	Feed	18	1 (5.56%)		
	Water	18	0 (0.00%)		
Chicken type	Layer	391	2 (0.51%)	0.7723	0.380
	Broiler	70	1 (0.51%)		
Age	Young	166	2 (1.20%)	1.2317	0.267
	Adult	295	1 (0.34%)		
Production system	Small scale	284	1 (0.35%)	1.0204	0.312
	Medium scale	177	2 (1.13%)		
History of *Salmonella*	Yes	353	1 (0.28%)	3.1472	0.076
	No	108	2 (1.85%)		
Use of antimicrobials	Yes	262	2 (0.76%)	0.1190	0.730
	No	199	1 (0.50%)		
History of *Salmonella* vaccination	Yes	82	1 (1.22%)	0.4990	0.480
	No	379	2 (0.53%)		
Fence	Present	361	2 (0.55%)	0.2409	0.624
	Absent	100	1 (1.00%)		
Footbath	Present	323	3 (0.93%)	1.2901	0.256
	Absent	138	0 (0.00%)		
Entrance of other animals	Yes	108	2 (1.85%)	3.1472	0.076
	No	353	1 (0.28%)		
PPE	Present	394	3 (0.76%)	0.5135	0.474
	Absent	67	0 (0.00%)		

PPE: Personal Protective Equipments

5. DISCUSSION

Salmonella is one of the major causes of heavy losses in chicken and foodborne diseases throughout the world (Sarba *et al.,* 2020). In the current study, the overall prevalence of *Salmonella* was 0.65%, and in cloacal swabs and feeds 0.47% and 5.56% respectively, but not identified from water samples. This prevalence of *Salmonella* was not comparable with the finding of (Eguale, 2018) who conducted on poultry farms in central Ethiopia and found a prevalence of 4.7% from fresh pooled fecal samples. This difference could be due to greater (549) samples and 48 farms studied than the current study. Similarly, this prevalence was not comparable with the findings of (Ali *et al.,* 2020), who found 14.6%, (Belachew *et al.,* 2021), isolated 24.30% and (Woyessa *et al.,* 2021) who found 18.4% prevalence in the fecal samples of chickens. And this difference might be the current study sites climates were colder than those study sites.

The result from cloacal swab agreed with the finding of (Aragaw *et al.,* 2010) who identified 0.8% from cloacal swab samples, and also prevalence of *Salmonella* in water sample (0.00%) agreed with the finding of (Shang *et al.,* 2018) and (Ayachi *et al.,* 2010), who also identified similar (0.00%) result. In another way, this result disagreed with the finding of (Abdi *et al.,* 2017) who got 14.8% from cloacal swabs; and they identified by biochemical tests only.

The questionnaire survey showed that most of the variables used to collect data were not associated significantly with the occurrence of *Salmonella* bacteria except the sample type variable which was statistically significant ($p < 0.05$). This may be due to the investigation of small number of *Salmonella* isolates. Even though there was no statistical significant association between the variables and the occurrence of the *Salmonella,* biosecurity measures and management activities had a great impact for the enterance (presence or absence) of the pathogen in the farm.

In the present study, the possible reasons for low *Salmonella* prevalence could be due to the fact that most of the chicken farms in this study had small number of chickens unlike large commercial chicken farms which holds thousands of chickens and the feeding and management activities associated with intensification allows for dissemination of the pathogen within the farm easily. The other possible reason might be due to mass administration of antibiotics to the chickens as

prophylaxis, growth promoter, and to increase production and others. The number of positive samples for *Salmonella* at Holeta was greater than the number of positive samples from Sebeta and Sululta, and this may be due to more number of farms were studied at Holeta than the two study sites and also it could be due to warmer climate of Holeta and Sebeta than Sululta town.

In this study, the isolates of *Salmonella* which were identified during the study were subjected to selected antimicrobial disks for the assessing of antimicrobial susceptibility tests. Accordingly, the 3 isolates including *Salmonella enterica* ST Gallinarum, *Salmonella enterica* ST Paratyphi B and *Salmonella* Enterica were assessed for 9 antimicrobial disks, and from these isolates *Salmonella enterica* ST Gallinarum was resistant to streptomycin and tetracycline. *Salmonella enterica* ST Paratyphi B and *Salmonella* Enterica were intermediate to meropenem and streptomycin disks. Even though researches which were directly conducted AST by identifying of *Salmonella* to species and subspecies level were rare in chickens in our country, this study was in agreement with the studies of (Woyessa *et al.,* 2021), and (Abunna *et al.,* 2017) by which most of the isolates were resistant to streptomycin and tetracycline.

6. CONCLUSION AND RECOMMENDATIONS

Salmonella is one of the Enterobactericeae family which causes a huge economic loss to the chicken industry, and have a major public health impact. The findings from this study showed that *Salmonella* can be present within the chickens and their environments including their feeds. According to this finding, Sululta town was conducive for chicken production than Holeta and Sebeta due to the absence of *Salmonella* isolate during the study. All of the isolates were confirmed by biochemical tests, identified by Omnilog test and *invA* virulent gene was detected within all of the three isolates of the identified *Salmonella.* These isolates were subjected to AST to selected disks and *Salmonella enterica* ST Gallinarum (*S*. Gallinarum) was resistant to streptomycin and tetracycline. *Salmonella enterica* ST Paratyphi B (*S*. Paratyphi B) and *Salmonella* Enterica were intermediate to meropenem and streptomycin disks. However, all of the isolates were 100% susceptible to ampicillin (AMP 10 μg), gentamicin (CN 10 μg), ceftriaxone (CRO 30 μg), amoxycillin-clavulanic acid (AMC 30 μg), sulphamethoxazole-trimethoprim (SXT 25 μg) and trimethoprim (W 5 μg). Imprudent use of antimicrobials in the farms can favor the continual occurrence of antimicrobial-resistant *Salmonella* within the animal population and environment. Since this bacteria can be transmitted to other animals and humans with their resistant genes, it can pose a risk of not to be cured by resistant developed antimicrobials.

In light of the above concluding remarks, the following recommendations were forwarded:

- Strong policy related to biosecurity should be developed and implemented for prevention and control of transmission of chicken Salmonellosis with their resistant genes.
- Awareness creation for chicken breeders on the importance of good chicken management, Salmonellosis prevention/control.
- Proper use of antimicrobials and control must be exercised and moreover appropriate regulation on the entrance of antimicrobials and its way of transportation, storage and others be executed.
- Removing of resistance developed antimicrobials from the market by all of the stakeholders is another key area of intervention to combat the alarming problem of antimicrobial susceptibility profiles. Drug of choice should be applied to clear resistant pathogen from the farms.

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8. ANNEXES

Annex 1: Procedures for preparation of different media

1. Buffered Peptone Water (BPW) (CM0509, UK)

20grams of dehydrated medium was added to 1 litre of distilled water. Mixed well and distributed into final containers. Then, it was Sterilized by autoclaving at 121°C for 15 minutes.

Composition (gram/litre):

Peptone 10.0, Sodium chloride 5.0, Disodium phosphate 3.5, Potassium dihydrogen phosphate 1.5, pH 7.2 ± 0.2 at 25°C.

2. Rappaport Vassiliadis Soya Broth (RVS Broth) media (HIMEDIA, M1491-500G, India)

Preparation:

27.11 grams of dehydrated medium was suspended in 1000 ml of distilled water. It was heated to dissolve the medium completely. Then, it was sterilized by autoclaving at 115°C for 15 minutes and dispensed into tubes.

Composition (gram/litre):

Papaic digest of soyabean meal 4.5, Sodium chloride 8.0, Potassium dihydrogen phosphate 0.6, Dipotassium phosphate 0.4, Magnesium chloride. hexahydrate 29.0, Malachite green 0.036, Final pH (at 25°C) 5.2±0.2.

3. Xylose-Lysine Deoxycholate Agar (XLD) Agar media (HIMEDIA, M031-500G, India)

Preparation:

58.68 grams were suspended in 1000 ml of distilled water. Next, it was heated with frequent agitation until the medium boiled. Then it was transferred immediately to a water bath at 45-50 °C. Mixed well and poured in to sterile petri plates.

Composition (gram/litre):

Yeast extract 3.0, L-Lysine 5.0, Lactose 7.5, Sucrose 7.5, Xylose 3.5, Sodium chloride 5.0, Sodium deoxycholate 2.5, Sodium thiosulphate 6.8, Ferric ammonium citrate 0.8, Phenol red 0.08, Agar 15.0, Final pH (at 25°C) 7.4±0.2.

4. Nutrient agar (Criterion, C6461, USA)

Preparation:

20.5gm of the dehydrated culture media was suspend in 1 liter of distilled water. Heated to boiled and mixed to be dissolved completely. Then, the medium was sterilized in the autoclave at 121°C for 15 minutes.

Composition (gram/litre):

Pancreatic digest of gelatin 5.0, Beef extract 3.0 and Agar 12.5 and Final pH (at 25°C) 6.8±0.2.

5. Methyl-Red and Voges-Proskauer (MR-VP, Oxoid, CM 0043, UK)

Preparation:

17grams the medium was weighed and added to 1 litre of distilled water. Mixed well, distributed into final containers and sterilized by autoclaving at 121°C for 15 minutes.

Composition (gram/litre):

Peptone 7.0, Glucose 5.0, Phosphate buffer 5.0 and Final pH 6.9 ± 0.2 (at 25°C).

6. Tiple Sugar Iron (TSI) Agar (Criterion, USA)

Preparation:

65.0 grams the medium was weighed and combined to 1 litre of deionized water. Mixed well thoroughly and boiled to dissolve completely. Sterilized by autoclaving at 121°C for 15 minutes. After autoclaved, slant tubes and allowed to cool, and final pH 7.3 ± 0.2 at 25°.

Composition (gram/litre):

Agar 12.0 gram, Casein peptone 15.0 gram, lactose 10.0 gram, sucrose 10.0 gram sodium chloride 5.0 gram, animal tissue peptone 5.0 gram, yeast extract 3.0 gram, beef extract 3.0 gram, dextrose 1.0 gram, ferric ammonium citrate 0.5 gram, sodium thiosulfate 0.3 gram, phenol red 24.0 gram

7. Simmons Citrate Agar (Biolife, Italy)

Preparation:

23.2 grams was suspended in 1 litre cold distilled water. Then, it was heated to boil with frequent agitation to dissolve completely. Distributed in to tubes and sterilized by autoclaving at 121 ^{o}C for 15 minutes and cooled in a slant position.

Composition (gram/litre):

Ammonium dihydrogen phosphate 0.2, Sodium ammonium phosphate 0.8, Sodium chloride 5, Sodium citrate 2, Magnesium sulphate 0.2, Brom thymol blue 0.08, Agar 15.

8. Peptone Water (PW, Titan, TM 330, India)

Preparation:

15 grams of the medium was dissolved in 1 litre of distilled water. The medium was heated gently to dissolve completely. Then, it was sterilized by autoclaving at 121 ^{o}C for 15 minutes and cooled to room temperature before use.

Composition (gram/litre):

Peptic digest of animal tissue 10.0 grams, sodium chloride 5.0 grams and its pH was 7.2 $\pm$ 0.2 at 25 ^{o}C.

9. Urea agar base (Oxoid, CM0053, UK)

Preparation:

2.4 grams was suspended in 95 ml of distilled water and dissolved completely by boiling. Then, it was sterilized by autoclaving at 115 ^{o}C for 20 minutes and cooled to 50°C and 5 ml of sterile 40% Urea Solution SR0020 was introduced aseptically. Mixed well and 10 ml amounts was distributed into sterile containers and allowed to set in the slope position.

Composition (gram/litre):

Peptone 1.0 gram, glucose 1.0 gram, sodium chloride 5.0 gram, di-sodium phosphate 1.2 gram, potassium dihydrogen phosphate 0.8 gram, phenol red 0.012 gram, agar 15.0 gram.

10. Mueller Hinton Agar (Criterion, C6421, USA)

Preparation:

38.04 grams of medium was combined with one liter of deionized water stirred to mix thoroughly. Then, it was dissolved completely by boiling and autoclaved at 121ºC for 15 minutes. Final pH: 7.3 ± 0.1 at 25 ºC.

Composition (gram/litre):

Agar 17.0 grams, casein acid hydrolysate 17.5 grams, beef extract 2.0 grams, starch 1.5 gram

Annex 2: Some of the media prepared and used during different tests for *Salmonella*

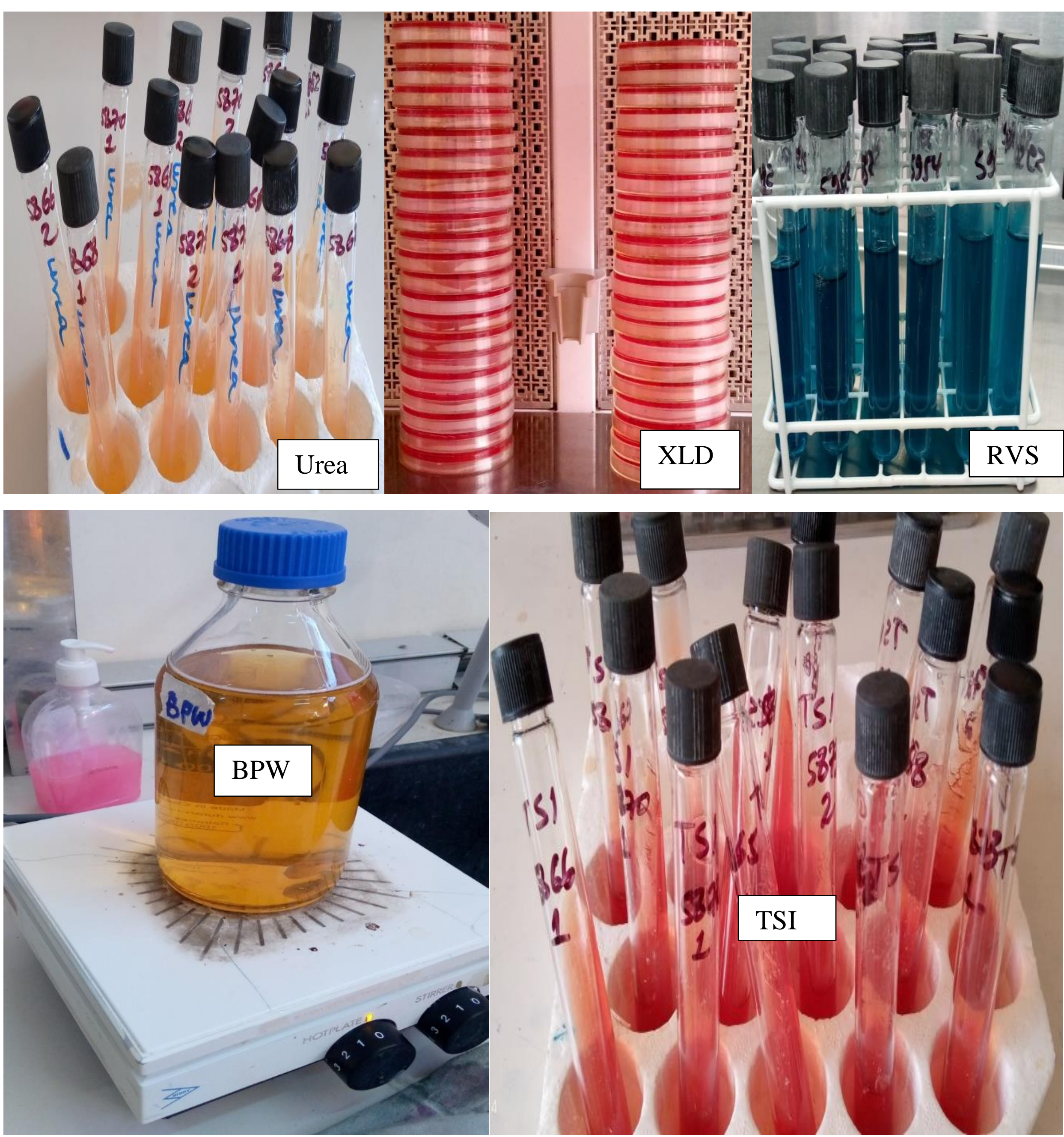


Figure 8: Some of the media prepared to conduct *Salmonella* tests

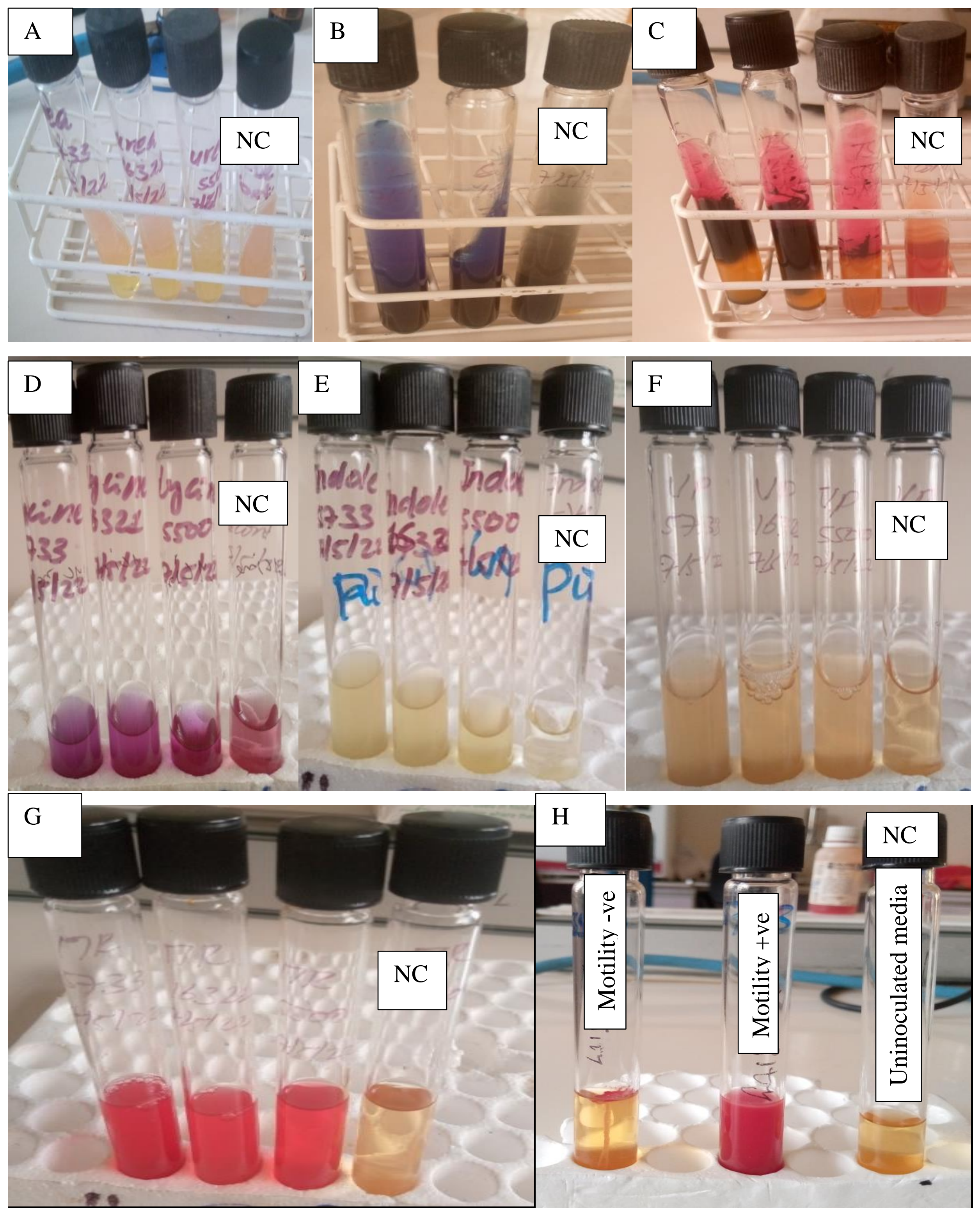


Figure 9: Biochemical and motility test results

A: Urea negative result with its negative control; B: Simmons citrate positive with its negative control; C: TSI positive with its negative control; D: Lysine test with its negative control; E: Indole negative result with its negative control; F: VP negative with its negative control G: MR positive with its negative control, H: Motility test results with its negative control; and NC: Negative Control.

Annex 3: Procedures for the identification of *Salmonella* by Biolog Microstation reader

Preparing of Samples

1) Isolation a Pure Culture
2) Culturing of *Salmonella* suspected sample on Biolog Universal Growth media (BUG) for 24 hours at 37 °C
3) Inocula Selection: Protocol A of inoculating fluid-A (IF-A) and Inoculum Density of 90-98% T was selected. Before starting, Gen-III MicroPlates and Inoculating Fluid-A (IF-A) were pre-warmed at room temperature.
4) Preparing of inocula:

- For each inocula preparation, blank the turbidimeter with the uninoculated inoculating fluid tube (wiped clean of dirt and fingerprints) by adjusting the 100% transmittance adjustment knob so that the meter reads 100%T.
- The target cell density should be in the range of 90-98%T for Protocol A.
- The cotton-tipped Inoculatorz was used to touch gently the top of a colony surface of the BUG agar plate.
- The swab shaft at the end was grasped, and held vertically; touch it to the cell growth.
- The Inoculatorz was inserted into the inoculating fluid tube and emulsified the organisms into the solution Read the turbidity in the turbidimeter
- This resulted in an approximate % transmittance range of 90 to 98 for Protocols A
- The GEN-III MicroPlates was inoculated with the suspension

5) Inoculating protocol

- The side of a MicroPlate with the organism was labelled.
- The cell suspension was poured into a multichannel pipette reservoir.
- Eight sterile tips were firmly attached to the 8-Channel repeating pipetter.

- The tips were filled with the suspension.
- All of the GEN-III MicroPlate wells were filled with 100 µL/well.
- The GEN-III MicroPlate covered with its lid.

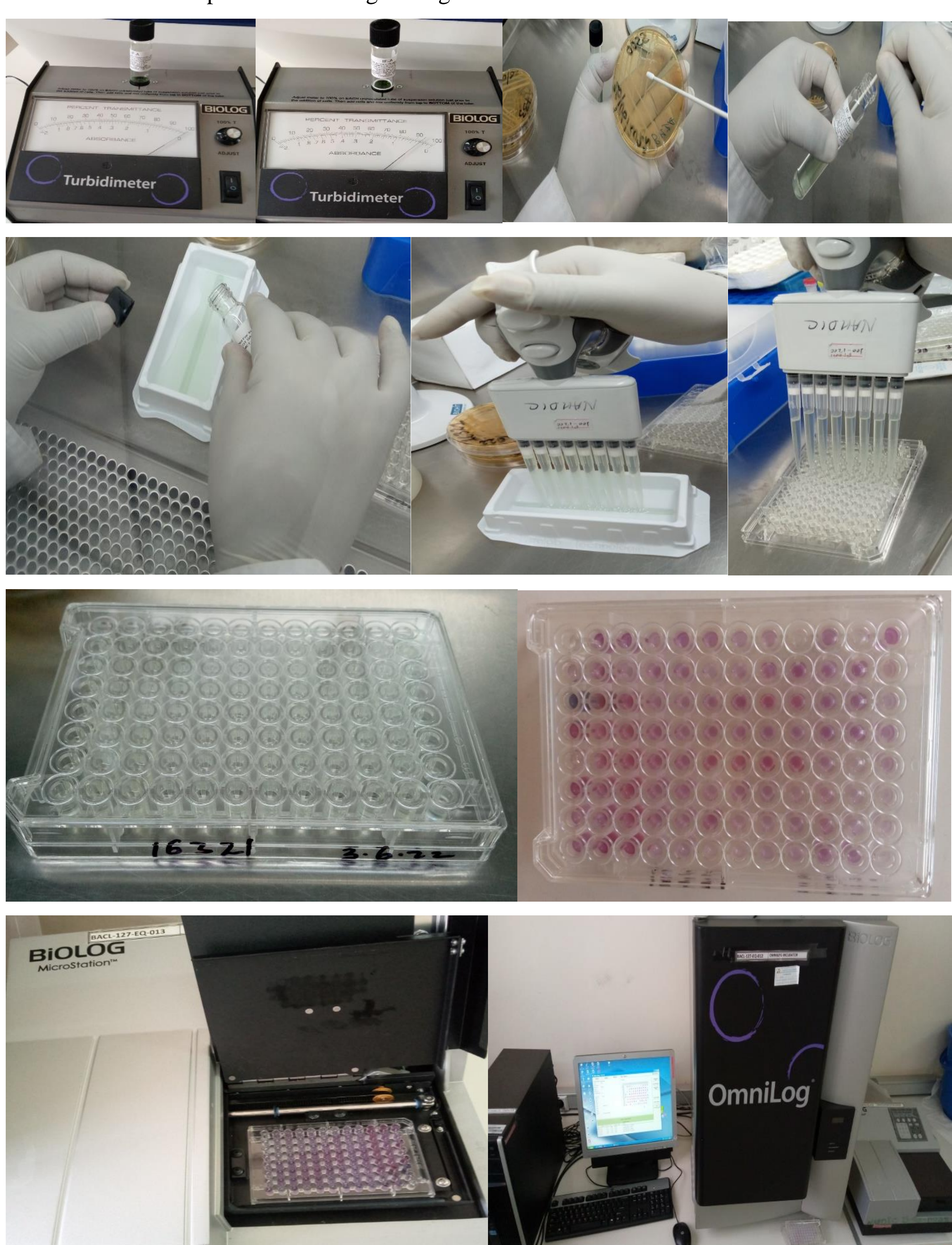
Incubating the GEN-III MicroPlates

- The MicroPlate was incubated at 33 °C for 22 hours

Loading and Reading MicroPlates

- After MicroPlates were inoculated, the information was entered into Biolog's Omnilog Data Collection software. This information related to organizing plate data in batches, managing files, and printing results. Then MicroPlates were you can loaded into the Omnilog incubator/reader and read

Annex 4: Activities performed during Biolog identification of *Salmonella*



Annex 5: Mastermix preparation for the amplification of invA gene

Components	**Amount (µL)**
Nuclease free water	8.5
iQ Multiplex powermix 2x	12.5
invA primer (forward and reverse)	1
invA Probe	0.5
Template (Colony dip)	2.5
Total reaction	**25**

Annex 6: Summary of Real Time PCR conditions and its result analysis

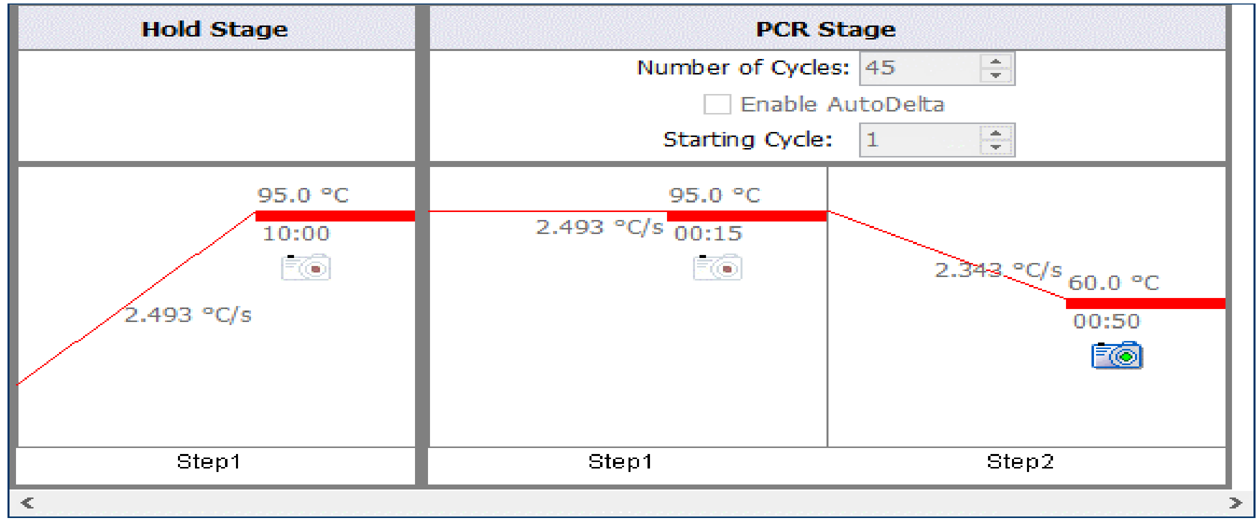


Figure 10: The real time PCR conditions at different temperatures with their respective times

Annex 7: Informed consent form

Addis Ababa University

College of Veterinary Medicine and Agriculture

Informed Consent Form

For <u>Chicken farm owners</u>Please specify <u>Livestock owners</u> (example: farmers/ abattoir workers/ butchers/ livestock owners, focus group discussant farmers etc)

After I introduce myself and explaining the scope and the objectives of the study in lay terms, the intended participant will be asked to take time to think if he/she may volunteer for a questionnaire survey, sample taking from them, or any intended study on their animals, properties etc (**specify depending on the type of the study**).

The participant:

- Is explained why he/she has been chosen to participate in the research while others are not.
  Because: Chicken farm owners are allowed their chicken farms to be visited, respond questionnaire and sample collection.

- Has got clear explanation on any possible benefits (individual or community) and harmful effects of the study on the participant, his/her animals or his property.
  Yes: Chicken farm owners are beneficial knowing the status, the economic effect and to know the *Salmonella* nature to control and prevent Chicken *Salmonellosis*.

- Is guaranteed that all information of private nature provided and his/her name will remain confidential.
  Yes: Information provided is confidential and will not be disklosed/ transferred to other party without the consent of the respondents.

- Is assured that if he/she does not understand some of the words or concepts of the intended work, he/she has the right to get adequate explanations all along the study time and has full right to withdraw from the study at any stage if he/she feels uncomfortable

  Yes: The right to get adequate explanations all along the study time and has full right to withdraw from the study at any stage if he/she feels uncomfortable.

Once, adequate explanations are made to the satisfaction of the intended participant, he/she will be asked to provide his/her informed consent as follows:

I have adequately understood the foregoing information (the objectives of the intended work, the activities to be undertaken, the benefits and harmful effects, my rights and obligations if I consent to participate, etc). I have had the opportunity to ask questions about it and any questions I have asked have been answered to my satisfaction. I consent voluntarily to be a participant in this study.

By signing on this sheet

Name of the participant/name of witness if participant is illiterate________________________

Signature ___________________ Date ____________________________

Or, **Verbally**: Name of Participant: **Chicken farm owners**

Reason for choosing verbal consent: **Questionnaire and sample collection**

Name of Researcher/person taking the consent: **EbisaMezgebu**

Signature ___________________ Date: ______________

Annex 8: Questionnaire Recording Format

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

Date: ________________

1. Owner name: ______________________________Phone number: ______________________

2. Farm name: ______________________Farm location: City/town: __________________

Woreda: _____________ Kebele: ___________Long: ______________ Lat: ________________

Flock size: _________ Age:_______________Layer/Broiler/Both: ____________

3. Farm production system: A) Small scale commercial B) Medium scale commercial

4. History of occurrence of Salmonellosis related clinical signs of disease in the flock:

A) Yes B) No

5. Did you use antimicrobial(s) for your flock? A) Yes B) No

5.1 If yes, for what purpose(s)? A) For disease control B) For disease prevention

C) To increase production D) A and B E) A and C F) B and C G) None

6. Did you vaccinate your flock with *Salmonella* Vaccine? A) Yes B) No

7. Biosecurity:

7.1 Fence A) Present B) Absent

7.2 Footbath A) Present B) Absent

7.3 Entrance of other birds/animals in to the farm: A) Yes B) No

7.4 Presence of PPE A) Present B) Absent

7.5 Presence of feeding/watering trough: A) Yes B) No

Annex 9: Ethical clearance letter

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: Certificate Ref. No: VM/ERC/09/04/13/2021

Name of Applicant: **Hika Waktole_ (BSc, MSc, Assit. Professor of Vet. Microbiology)**

Address: **Department of Microbiology, immunology and Vet. Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University**

Title of the project: *Biosecurity practices in Poultry Farms: isolation, identification and molecular characterization of major bacterial pathogens, investigation of major bacterial zoonosis and biosecurity based interventions towards enhancing production efficiency and profitability in poultry farms in central Ethiopia*

Date of application: **March, 2021**
Nature of the project: **Mildly invasive /little stress**
Target animal species: **Domestic chicken**
Number of animals involved: **5760**
Study area: **Central Ethiopia, Ethiopia**

Minutes No. and date of review: **VM/ERC/04/13/021, 21/04/2021**

The above mentioned research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary
1. Any major study on human subjects (except questionnaire survey) should get a separate clearance from relevant bodies

Getachew Terefe (DVM, PhD)
Chairman

Signature

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Please quote Our Ref. No. When replying

ፋክስ Fax 251-11-4339933 | ስልክ Tel. +251 114338450 | P.O. Box 34 | ቢሾፍቱ፣ ኢትዮጵያ Bishoftu, Ethiopia