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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

MVSc THESIS

ISOLATION, MOLECULAR DETECTION, ANTIBIOGRAM PROFILE AND THE ASSOCIATED RISK FACTORS OF SALMONELLA FROM POULTRY FARMS IN AND AROUND DEBIRE BIRHAN, CENTERAL ETHIOPIA

BY

YONAS AYELE

AUGUST, 2021

BISHOFTU, ETHIOPIA

ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

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A thesis submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Veterinary Science in Veterinary Microbiology

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: Yonas Ayele entitled 'Isolation, molecular detection, antibiogram profile and the associated risk factors of *Salmonella* from poultry farms in and around Debire Birhan' and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Veterinary Science in Veterinary Microbiology.

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LIST OF ABBREVIATIONS

AE	Elution Buffer
AST	Antimicrobial Susceptibility Test
AW	Washing Buffer
BGA	Brilliant Green Agar
CDC	Centers for Disease Control
CLSI	Clinical Laboratory Standards Institute
HACCP	Hazard Analysis Critical Control Point
IMViC	Indol-Methyle Red-Vagos Prosker and Citrate Utilization Test
ISO	International Organization for Standardization
LIA	Lysine Iron Agar
MDR	Multidrug-Resistant
MIC	Minimal Inhibitory Concentration
MKTTn	Muller-Kauffmann Tetrathionate-Novobiocin
m-PCR	Multiplex Polymerase Chain Reaction
MR-VP	Methyl–Red Voges Proskauer
NTS	None Typhoidal Salmonella
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PBW	Buffered Peptone Water
RVS	Rappaport Vassiliadis
SCVs	*Salmonella* Containing Vacules
SDS	Sodium Dodecyl Sulfate
SGI	*Salmonella* Genomic Islands
SM-ID	*Salmonella* identification
SPI	*Salmonella* Pathogenicity Island
Spv	*Salmonella* Plasmid Virulence
T3SS	Type Three Secretion System
TAE	Tris-Acetate EDTA
TSB	Typtone Soya Broth

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STATEMENT OF THE AUTHOR

First and foremost, I confirmed that this research is entirely my own work, and that all sources of information used in this study have been properly recognized. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University's College of Veterinary Medicine and Agriculture, and it has been deposited in the College library to be made available to borrowers in accordance with the Library's rules. I solemnly declare that I am not submitting my thesis to any other institution anywhere for the award of any academic reward.

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ABSTRACT

Salmonella infections are very common in both animals and humans that cause significant economic and public health impacts in Ethiopia. A cross-sectional study was conducted from December 2020 to June 2021 to isolate, perform molecular detection, determine antibiogram, and asses the associated risk factors of *Salmonella* species from poultry farms in and around Debire Birhan, Central Ethiopia. For these purposes, a total of 384 samples obtained from cloacal swab (n=136), fecal dropping (n=130), chicken feed (n=64), and drinking water (n=54) were aseptically collected and examined. Out of 26 poultry farms subjected to standard bacteriological culture method, 19 (73.07%) were found positive for *Salmonella* isolates at least in one of the examined sample types. The overall bacteriological prevalence of *Salmonella* species isolated was 14.06% out of the total 384 samples analyzed. Among the determinants, sample type and flock size were strongly associated with the isolation and identification rate of *Salmonella* (P<0.05). Accordingly, higher isolation and identification rates found in fecal droppings 29(22.30%) and flock size greater than 1500 chickens/farm 20(23.25%), respectively. However, the isolation rate was not affected by location, age, and breed of the chickens. The molecular detection rate of *S*. Typhimurium was 50% out of the 30 *Salmonella* isolates subjected to a conventional polymerase chain reaction. The detection rate of *S*. Typhimurium showed significant association with age groups (p=0.03) and flock size (p=0.04) where higher isolation rates were recorded in the age group greater than 18 weeks and flock size greater than 1500 chickens. The disk diffusion antimicrobial susceptibility finding showed that all *S*. Typhimurium isolates were found multidrug resistant and higher antimicrobial resistance observed to ampicillin (93.3%) followed by oxytetracycline (86.7%), sulfamethoxazole (46.7%), and tetracycline (40%). On the other hand, 100% and 73.3% of isolates were susceptible to ciprofloxacin and gentamycin, respectively. In nut shell, the present study disclosed higher isolation and detection rate of *Salmonella* species and also the appearance of multidrug resistant *S*. Typhimurium to several drugs necessitating the urgency for further detailed molecular characterization to come up with the circulating *Salmonella* serovars and antimicrobial resistance strains and responsible genes.

Keywords: *Antimicrobial resistance, Chickens, Debere Birhan, Detection, Isolation, PCR, Salmonella* Typhimurium

1. INTRODUCTION

Poultry production is an integral part of livestock production that has a peculiar privilege to contribute an important role in the national economy (Goitom, 2017). Ethiopia has great potential to expand modern poultry production and promising economic development of the country. However, poultry production is constrained by traditional technologies, high disease prevalence, inadequate supply of imputes (feed and veterinary drugs), low genetic potential of breeds and poor managemental practices (Fitsum, 2014). Among these, infectious diseases are remaining the major health constraints that hindering their intended potential (Endris *et al.*, 2013; Melese, 2015).

Salmonellosis is one of the most important bacterial diseases in the poultry industry and other avian species causing heavy economic loss due to high morbidity, mortality, and impaired productions (Cheraghchi *et al.,* 2014). *Salmonella* spp. accounts for the major food-borne disease in most countries of the world, especially of in developing countries (Sedeik *et al.*, 2019). According to national and global epidemiological registries, *Salmonella* species is considered as one of the principal causes of foodborne bacterial diseases in humans. Avian salmonellosis occurs in chickens by one or more serovars of the genus *Salmonella*, under the family *Enterobacteriaceae* (Martelli and Davies, 2012; Dmc *et al.*, 2016).

Salmonella is the general name used for a group of more than 2600 different species bacteria under the family *Enterobacteriaceae* (Ryan *et al.*, 2017). The bacterium was first isolated by the two American veterinarians, Salmon and Smith, from pigs which causing hog cholera and usually exists as a harmless communal. However, pathogenic *Salmonella* strains cause a variety of diseases in both animals and humans (Angela *et al.*, 2009). According to latest nomenclature, the genus *Salmonella* consists of two species *Salmonella enterica (S. enterica)*, *Salmonella bongori* (*S. bongori)* based on the differences in their 16S rRNA sequence analysis (Ryan *et al.*, 2017). Based on their genomic relatedness and biochemical properties, the type species, *S. enterica*, further classified into six subspecies namely, (I) *S. enterica* subsp. *enterica;* (II) *S. enterica* subsp. *salamae;* (IIIa) *S. enterica* subsp. *arizonae*; (IIIb) *S. enterica* subsp. *diarizonae (*IV) *S. enterica* subsp. *houtenae*; and (VI), *S. enterica* subsp. *indica*. *S. enterica* subsp. *enterica* is predominant subsp. in mammals and contributes approximately 99% of *Salmonella* infection (Angela *et al.*, 2009; Eng *et al.*, 2015).

The genus *Salmonella* comprises rod shaped, facultative anaerobe, non-spore-forming gram-negative organisms. Members of the genus *Salmonella* are motile by peritrichous flagellation except for *S. enterica* serovar Pullorum and *S. enterica* serovar Gallinarum, which lacks flagella (Michael and Schwarz, 2016). Biochemically the genus *Salmonella* characterized as positive for catalase, methyl red, Simmons citrate and ferment sugar with producing H_2S except *S.* Typhi, while negative for oxidase, indole and Voges Proskauer (VP) (Cheraghchi *et al.*, 2014; Michael and Schwarz, 2016; Djeffal *et al*., 2018).

Based on diseases they cause, *Salmonella* serovars are classified into two groups. The first group consists of *Salmonella* Pullorum (*S.* Pullorum) and *Salmonella* Gallinarum (*S.* Gallinarum) which are characterized as non-host adapted, pathogenic, non-motile, and causes pullorum disease and fowl typhoid in chickens, respectively. The nontyphoidal *Salmonella, Salmonella* Typhimurium *(S.* Typhimurium), and *Salmonella* Enteritidis (*S.* Enteritidis) are the second groups of *Salmonella* which are known by their motility and responsible for sub clinical Salmonellosis in laying chickens and produces human infection (Mayah, 2011; Ababa, 2013; Cheraghchi *et al.*, 2014; Dmc *et al*., 2016; Mesfin and Dejene, 2017).

The laboratory detection of *Salmonella* both in the human and animal health sectors is crucial to identify circulating *Salmonella* serovars. Laboratory identifications are mainly targeted to detect viable *Salmonella* organisms and their antigenic components. Several methods have been employed for the diagnosis of salmonella infections include: standard bacteriological culture methods, immunological serotgrouping and nucleic acid detection (Mohammadzade *et al.*, 2017). Nowadays, identification of *Salmonella* through standard conventional culturing complemented with DNA based molecular techniques have been employed (Cheraghchi *et al*., 2014; Naik *et al*., 2015). The Various *Salmonella* serotypes have been identified using serotype specific genes using conventional PCR (Carrique Mas and Davies, 2008; Asif *et al*., 2017; Mohammadzadeh *et al*., 2017).

Salmonella infection has been reported in almost all industrialized and developing countries, with varying degree of prevalence. However, it appears to be very common in developing countries (Nair *et al.,* 2015). Previous researches in Ethiopia have clearly indicated that different *Salmonella* serovars have been detected in various regions of the country, which have been isolated form humans, animals, food of animal origin as well as their environment. The frequent occurrence of Salmonella infection in Ethiopian poultry farms plays significant social and economic impacts due to its high morbidity and mortality rate, production loses and cost of treatment, prevention, and control (Kassaye *et al.*, 2015; Ayalew *et al.*, 2017; Asfaw *et al.*, 2020). As most strains of *Salmonella* are potentially pathogenic to humans and animals, infections due to none typhoidal *Salmonella* is considered as very common and have an important public health impact in many parts of the world (Singh *et al.*, 2010; Nair *et al.*, 2015). Among *Salmonella* strains, serovar enteritidis and typhimurium were predominantly isolated from food of animal origin including chickens and cause human salmonellosis (Asif *et al.*, 2017).

An increase in resistance of *Salmonella* to one or multiple antimicrobials has been identified in both public health and veterinary sectors in Ethiopia. In this respect, the majority of the *Salmonella* isolates from poultry farms and poultry products in the previous studies were found to be resistant to several antimicrobials. The irrational use of first line drugs and close contact between animals and humans have supposed the primary causes for the development of multipledrug resistance which could pose a serious problem in the near future in Ethiopia (Chanie *et al.,* 2009, Eguale, 2018).

Currently, Debire Birhan is known by the highest laying chicken distributor to 24 districts of North Shoa Zone (Mengsite *et al.*, 2019). For that reason, there has been a tremendous development of small and medium scale poultry production. However, there is no available information about the status of salmonella infection, serotypes circulating in the area, and antimicrobial resistance profile in chickens. Therefore, the present research was initiated and designed with the aim of exploring the status of salmonella infections in chicken farms in and around Debire Birhan, Central Ethiopia.

General objective

- The general objective of this study was to carry out isolation, molecular detection, determinination of the antibiogram profile and assessment of associated risk factors of *Salmonella* species from chickens and samples obtained from surroundings of the poultry farms in and around Debre Birhan, Central Ethiopia.

Specific objectives

- To isolate and identify the *Salmonella* species from chickens and their environment
- To detect *Salmonella* strains circulating in the study area through conventional PCR.
- To determine the antibiogram profile of of *Salmonella* isolates
- To assess the associated risk factors for the occurrence of *Salmonella* in chickens

2. LITERATURE REVIEW

2.1. Taxonomic Classification of *Salmonella* Spices

The genus *Salmonella* belongs to the family *Enterobacteriaceae* and consists of two species, namely *Salmonella bongori* (*S.* bongori) and *Salmonella enterica (S. enterica)* based on differences their 16S rRNA sequence analysis (Eng *et al.*, 2015; Ryan *et al.*, 2017). Unlike *S. bongori,* which contains less than 10 extremely rare serovars, *S. enterica* contained more than 2600 serovars and is divided into six subspecies: (I*) Salmonella enterica subsp. Enterica, (*II*) Salmonella enterica subsp. salamae (*IIIa*) Salmonella enterica subsp. Arizonae, (IIIb) Salmonella enterica subsp. Diarizonae,* (IV) *Salmnella enterica subsp. Houtenae, and (VI) Salmonella enterica subsp. Indica* based on their biochemical characteristics. Of these subspecies of *Salmonella, S. enterica subsp. enterica* is the most commonly found and attributes about 99% of *Salmonella* infections both humans and animals. On the other hand, *S. bongory* and subsp. of *S. enterica* other than *S. enterica subsp. enterica* is commonly found in cold blooded animals and the environment (Jajere, 2019).

The Kauffman-White classification method also further classifies *Salmonella* spp. into serotypes based on antigenicity of lipopolysaccharide antigen (O antigen), flagellar protein antigen (H antigen) and sometimes the capsular (VI) antigens. Accordingly, there are above 2600 serotypes of *Salmonella* and new serotypes are listed on annual updates of the Kauffman White scheme (Grimont and Weill, 2007; Ryan *et al.*, 2017). Agglutination made by numerous O antigens and their specific antibodies is used to classify *Salmonella* spp. into the following serogroups: A, B, C1, C2, D, and E. (Liu *et al.*, 2010 and 2014).

Salmonella H-antigens are heat labile proteins associated with peritrichous flagellar antigens (H-antigens). flagellar antigens (H antigens) have been thoroughly documented, and they are primarily encoded by one of two genes, *fliC* and *fljB*, which express the phase 1 H antigen and the phase 2 H antigen, respectively. Phase 1 H antigens are specific for certain serotypes that determine the immunological identity of distinct serovars, whereas phase 2 antigens are non-specific antigens that contain diverse antigenic subunits proteins that can be shared by many serovars. *Salmonella* serovars also produce capsular antigens (K-antigens), a heat sensitive surface-bound polysaccharide in which their expression is activated after the invasion of epithelial cells (Yousef and Carlstrom, 2000; Nalbantsoy *et al.*, 2010; Mcquiston *et al.*, 2011).

Salmonella spp. can be classified as host restricted, host adaptable, or none host restricted based on their host-pathogen interaction. *Salmonella* serovars that infect specific hosts are classified as host-restricted *Salmonella* (*S.* Typhi and *S.* Paratyphi infect humans only; *S.* Gallinarum and *S.* Pullorum infect poultry only). The host adapted *Salmonella* group adapted for particular species of animals and also pathogenic to humans. For instance *S.* Abortusovis in sheep, *S.* Dublin in cattle, *S.* Choleraesuis in swine and *S.* Abortusequi in horse. Serovars Typhimurium, Enteritidis, and Heidelberg are none host specific *Salmonella* strains that can infect both animals and humans (Jay, 2000).

Salmonella Gallinarum *(S.* Gallinarum) and *Salmonella* Pullorum *(S.* Pullorum), biovars of *Salmonella enterica* subsp. *enterica* serovar Gallinarum, are host-specific *Salmonella* for avian species which cause fowl typhoid and pullorum disease, respectively, and resulting in huge economic losses to the poultry industries every year (Geetha and Palanivel, 2018). *S.* Typhimurium and *S.* Enteritidis, the most prevalent serotypes of this bacteria, cause illnesses in people and animals *(*Mohammadzadeh *et al*., 2017).

2.2. Morphology and Growth Requirements of *Salmonella* species

The Genus *Salmonella* obey the features of the family *Enterobacteriaceae:* they are straight rod gram-negative bacilli, non-spore forming, facultative anaerobes, non-capsulated (except *S.* Typhi, *S.* Paratyphi C, and some strain of *S.* Dublin) and having size 0.2-1.5×2-5 µm. Members of the genus *Salmonella* are motile by the means of flagella except for biovars *S.* Gallinarum and *S.* Pullorum (Geetha and Palanivel, 2018; Jajere, 2019).

Salmonella can grow and multiply under various environmental conditions outside the living hosts (non-fastidious). They grow with little (0.4-4%) or in the absence of sodium chloride (Jajere, 2019). *Salmonella* can adapt a wide pH range from pH 3.8 to pH 9.5 with the optimum pH requirement for their optimal growth ranges from 6.5-7.5. The acid tolerance response by *S.* Typhimurium enables the cell to be protected against acidic stress and can survive at a pH level of 3 to 4, but is activated when the pH increased to 5.5 to 6.0. However, pH above 9.0 and below 4.0 may have bactericidal effect (Ryan *et al.*, 2017).

The rapid growth rate of *Salmonella* spp. has been recorded in the temperature range of 25 and 43^{0}C. However, the growth of *S.* Typhimurium and other *Salmonella* spp can grow at 6.2^{0}C and 45^{0}C, respectively have been reported. The optimal moisture content required for

Salmonella ranges between 0.99 and 0.94 although it can survive the moisture content less than 0.2 such as in dried foods. *Salmonella* is sensitive to heat and will not survive temperatures above 70^{o}C, but resistance to drying like in dried feces and dust has been shown. In contrast to this, *Salmonella* spp. can also survive in water for a prolonged time (Geetha and Palanivel, 2018).

2.3. Cultural and Biochemical Characteristics of *Salmonella* species

Salmonella can grow on a large number of bacteriological culture media and produce visible and distinctive colonies within 24 hours at about 37^{0}C. Sub lethally injured *Salmonella* cells can be recovered in none selective and selective enrichment media, commonly buffered peptone water (BPW) and Rappaport-Vassiliadis (RV), respectively. Both selective and differential plating media like Xylose Lysine Deoxycholate Agar (XLD), Brilliant Green Agar (BGA), *Salmonella Shigella* Agar (SSA), and Bismuth Sulfite Agar (BSA) allow the growth of *Salmonella* organism, while at the same time suppressing the growth of other bacteria. For instance, on XLD and BGA, most *Salmonella* serovars appear as colorless colonies with a black center and spherical moist colonies with purple color, respectively (Lee *et al*., 2015; Ryan *et al.*, 2017).

Distinctive biochemical reactions of *Salmonella* spp. are the classical features in phenotypic characterization of clinically important *Salmonella* spp. Except for *S*. Choleraesuis, *S*. Typhi, and *S*. Paratyphi, most *Salmonella* serotypes utilize citrate. Other prominent characteristics of this genus include the ability to reduce nitrate to nitrite, ferment glucose, dextrose, maltose, and mannitol with the production of acid and gas, but cannot ferment lactose and sucrose. Almost all serotypes do not produce indole, negative for Voges Proskauer reaction, decarboxylate lysine, and produce hydrogen sulfide. Most veterinary important *Salmonella* serovars utilize dulcitol except *S. enterica* subsp. *arizonae* (IIIa) and *S. enterica* subsp. *arizonae* (IIIb) (Geetha and Palanivel, 2018).

2.4. Epidemiology of Salmonellosis

2.4.1 Host range and geographic distribution

Serovars of the type subspecies, *Salmonella enterica* subsp. *enterica* are responsible for most *Salmonella* infections both in humans and domesticated animals (Uzzau *et al*., 2001). Animals like cattle, swine, poultry, wild birds, and pets are the primary hosts for non-typhoidal *Salmonella* (Nair *et al.,* 2015). The host range of *Salmonella* serovars could be host specific, host adapted, and none host specific serovars. Host-specific *Salmonella* serovars are associated with only one particular host species (e.g., *S.* Typhi in humans and *S.* Gallinarum in poultry). Host adapted *Salmonella* serovars are primarily associated with a particular host, but they can also cause disease in other animal species (e.g., *S.* Dublin in cattle and *S.* Choleraesuis in pigs). None host-specific *Salmonella* serovars (e.g., *S.* Enteritidis *and S.* Typhimurium*)* induce an asymptomatic carrier state in a broad range of animal species and are also associated with humans. Compared to others, host-adapted *Salmonella* serovars are characterized by a wider geographical spread (Martelli and Davies, 2012).

Biovar Pullorum (*S.* Pullorum) and Gallinarum (*S.* Gallinarum) are considered to be highly adapted to birds and cause the most important diseases of poultry namely pullorum disease and fowl typhoid, respectively. Pullorum disease is usually symptomatic to the first 2-3 weeks of age and occasionally occurs in adults. However, both growing and mature chickens are probably most susceptible to fowl typhoid. In recent years research findings revealed that infection due to Salmonella serovars is increasing continually in breeder flocks, commercial broiler, and layer flocks (Geetha and Palanivel, 2018; Bishwabidyalay *et al.*, 2019).

The worldwide distribution of fowl typhoid and pullorum disease has significant economic and public health importance. They have commonly distributed in Africa, the Middle East, the Indian Subcontinent, South America, and perhaps other continents of the world. However, most countries in Europe, U.S., Canada, Japan, and New Zealand, have highly developed poultry industries, have eradicated these diseases. None typhoidal *Salmonella* Serovars (*S.* Enteritidis and *S.* Typhimurium) have been frequently reported in Africa. In Ethiopia, the epidemiological pattern of Salmonellosis differs greatly between geographic areas mainly depending on climate, population density, and food harvesting and processing technologies (Lutful Kabir, 2010; Eng *et al.,* 2015).

2.4.2 Reservoir and source of infection

Major reservoirs for the genus *Salmonella* are man, farm animals, pigeons, waterfowl, and wild birds. Potential reservoirs like rodents, pests, and insects can perpetuate salmonella infection (Hafez, 2016). Visitors, feed and chicken buyers who move from house to house and farm to farm, may also carry infection. The majority of *Salmonella enterica* serovars are host adapted and can infect and cause clinical or subclinical infections in asymptomatic animals refer to as “carriers”. These animals are an important source of contamination of the poultry environment and animal products at the harvesting stage. Carrier hosts perpetuate the transmission of *Salmonella* due to continuous or intermittent shedding of this organism in their feces without showing any clinical signs. They are also carried latently in the mesenteric lymph nodes without shedding but can become reactivated following stress or immune suppression (Jajere, 2019).

2.4.3 Mode of Transmission

Infected chickens are sources of organisms; they infect other animals directly or indirectly by contaminating the environment, primarily feed and water supplies by their excreta. The principal reservoir of these bacteria in chicken is its intestinal system, which is contaminated by eggs in the cloacal region via a horizontal pathway. Horizontal transmission is mediated by direct bird-to-bird contact, ingestion of contaminated food stuff, water, contact with contaminated equipment and personnel. Within a flock, healthy chickens may also acquire the disease through pecking of contaminated soil or litter, cannibalism of infected birds, wounds on the skin, and eating of infected eggs (Djeffal *et al*., 2018; Rahman *et al.,* 2018).

Trans ovarian transmission in infected chickens is another important route of contamination that leads to egg borne Salmonellosis (Singh *et al*., 2010). *Salmonella* from infected breeder flocks transmit to their progeny via the egg and even to chicks hatched from eggs laid by infected progeny. *Salmonella* from infected eggshells can penetrate shells during incubation and causes vertical transmission. Host restricted *Salmonella* serovars, such as *S.* Gallinarum and *S.* Pullorum are known by vertical transmission, but it has also been demonstrated in non-host-specific *Salmonella* like *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg. In general transmission between farms is due to poor biosecurity measures (Rahman *et al*., 2018).

2.4.4 Pathogenicity and virulence factors

The pathogenicity of *Salmonella* serotypes differs depending upon the difference in virulence potential of serovars and susceptibility of an infected host. The pathogenesis of *Salmonella* infection is governed by many virulence factors include type three secretion systems (T3SS), virulent plasmids, flagella, capsule, and other adhesion systems. Parts of adhesion systems produced by most serovars of *Salmonella enterica* include adhesins, invasins, toxins, fimbriae, and hemagglutinins. These virulence factors enable *Salmonella* to colonize the host cell and bypassing the host’s defense mechanisms. Genes contained on a wide range of genetic elements, including bacterial chromosomes, plasmids, prophages, and other SPIs, encode these bacterial virulence factors (Jajere, 2019b).

Type III secretion system

The virulence of *Salmonella* is determined by their capacity to penetrate host cell immune defense mechanisms and withstand the intracellular degradation effects of phagocytic cells and the complement system. Therefore, the invasive properties, intracellular replication, and survival particularly within the macrophage are the pathogenicity determinant factors of *Salmonella.* These factors are highly linked to the ability of this organism to induce systemic infection by disrupting innate immune responses. The *Salmonella* pathogenicity islands encode a functional type three secretion system (T3SS), responsible for these properties. T3SS serves as a 'molecular syringe' that translocates bacterial toxins and other effector proteins from the bacterial cytoplasm to the host cell membrane. Several effector proteins are secreted through T3SS involved in *Salmonella* adhesion, invasion, and toxicity (Foley *et al.*, 2013; Jennings *et al.*, 2017).

All serovars of *S. enterica* contain two genes known as *Salmonella* Pathogenicity Island one and two (SPI-1 and SPI-2), gene clusters located at the large chromosomal DNA region. T3SS translocate effectors molecules across the cell membrane of host cells and stimulate invasion of epithelial cell line, induction of intestinal secretion, localized inflammation, and intracellular survival within the macrophage. The SPI-2 T3SSs in *Salmonella* harbors genes that are responsible for secreting effector proteins in *Salmonella* containing vacuoles (SCV). SPI-I T3SS also limits the host immune response to infected cells through modulation trafficking of the SCV to avoid fusion with the lysosomes and suppress antigenic presentation in dendritic cells. *Salmonella* lacking a functional SPI-I type three secretion system unable to invade epithelial cells, and induce cytokine synthesis which intern inhibits inflammatory activities (Pathol, 2002; Foley *et al.*, 2013; Eng *et al.,* 2015; Jennings *et al.*, 2017).

Virulence plasmids

Virulence factors of most enteric bacteria such as Salmonell*a, E. coli, Yersinia,* and *Shigella* species are often encoded by plasmids and are responsible for their pathogenicity. Among the serovars of *Salmonella enterica* subsp. *enterica, S.* Gallinarum/Pullorum, *S.* Typhimurium, *S.* Enteritidis, *S.* Dublin, *S.* Choleraesuis, and *S.* Abortusovis are known to harbor virulence plasmids. *Salmonella* plasmid virulence (spv) locus found in the plasmid contains five genes designated spv RABCD which play significant role in the multiplication of *Salmonella* in the reticulo-endothelial system, including the liver and spleen (Jajere, 2019b). An experimental study on zebra fish larvae showed that *S.* Typhimurium containing spv operon display an increased bacterial survival by suppressing the function of defense cells, neutrophils, and macrophages (Puente *et al.*, 2017). Other loci of the plasmid play a significant role in other stages of the infection process. *Salmonella* serotypes' antimicrobial resistance to one or more antibiotics is also due to a plasmid containing virulence genes (Eng *et al.*, 2015).

Endotoxins and exotoxins

Salmonella can produce both endotoxins and exotoxins. The endotoxin, which is the lipid portion (lipid A) of the outer membrane lipopolysaccharide (LPS) of *Salmonella*, elicits a diversity of biological responses. The cytotoxin, a type of exotoxins, is also significant virulence factors for *Salmonella* defined as their ability to kill mammalian cells and inhibit protein synthesis. Most Strains of *S.* Choleraesuis, *S.* Enteritidis, and *S.* Typhi produce a heat-labile, trypsin sensitive cytotoxin which is not neutralized by antibodies. The second type of exotoxin called enterotoxins can produce mucosal damage in the cell (Hitchcock *et al.*, 1986). Salmonella enterotoxin and salmolysin encoded by *stn* and *slyA* genes are the two other types of exotoxins and determine hemolytic properties. *Salmonella* serovars namely *S.* Typhi, *S.* Enteritidis, and *S.* Typhimurium are known to produce these toxins (Van Asten and Van Dijk, 2005; Jajere, 2019b).

Super oxide dismutase

One of the host immune defense mechanisms against microbial infection is the production of reactive oxygen species that are required for the killing of intracellular pathogens. However, the bacteria evade the normal killing mechanisms of these phagocytic cells using a variety of virulence factors. *Salmonella c*an produce periplasmic superoxide dismutase capable of catalyzing the conversion of superoxide radicals to hydrogen peroxide. This degradative process enables this pathogen to survive and propagate in macrophages and causes serious systemic diseases. For example, *S.* Typhimurium strain 14028 produces two periplasmic superoxide dismutases, SodCII and SodCI. SodCI and other closely related bacterial SodCs have developed to bind peptidoglycan and greater binding affinity of SodCII to peptidoglycan confers the ability to contribute to resistance to phagocytic superoxide (Slauch, 2011; Tidhar *et al*., 2016; Rhen, 2019).

Fimbriae and flagella

Salmonella has strain-specific surface proteins that can colonize and adapt to a variety of environmental conditions. Fimbriae, also called pili, are filamentous adhesive appendages on the bacterial surface that play a role in pathogenesis by serving as surface adhesion factors. The structural subunits of exported and assembled in a specific ordered manner on bacterial surface and serves as a source of diversity for *Salmonella* serovars (Yue *et al.*, 2012; Jajere, 2019b). Each type of fimbria is decoded from genes that grouped in one cluster, sometimes together with fimbrial expression regulator gene(s). Most serovars of *Salmonella* possess 12 fimbrial gene clusters that encode for structural, assembly, and sometimes regulatory proteins required for the production of bacterial surface adherence structures. Biofilm production, cellular invasion, haemagglutination, macrophage interactions, and host adaptability during bacterial infection are all important functions of fimbriae (Nuccio and Bäumler, 2007; Foley *et al*., 2013).

Flagella, located on the surface of certain *Salmonella* serovars, are a source of flagellin phase variation, which creates phenotypic heterogeneity of the flagellar antigens (H antigens). The majority of *Salmonella* serotypes possess about 5 to 10 seemingly randomly positioned flagella. The well studied *Salmonella* serotype, *S.* Gallinarum, and *S.* Pullorum are annotated as non motile, non flagellated. Most *Salmonella* spp. contains two distinct genes namely *fliC* and *fliB* responsible for encoding phase one and phase two flagellin proteins respectively. The *fliβ* gene is present in all *Salmonella* and has homologues in other enteric bacteria. The *fljC* gene, on the other hand, is unique in *Salmonella* enterica and is found in four subspecies of these bacteria (subspecies I, II, IIIb, and VI). According to the phase variation mechanism, diphasic serotypes express two forms of flagellin, while monophasic serotypes express just one type of flagellin at a time (Mcquiston *et al.*, 2011). The virulence factor flagella provide motility, host cell surface adhesion, and biofilm formation (Van Asten and Van Dijk, 2005; Eng *et al.*, 2015).

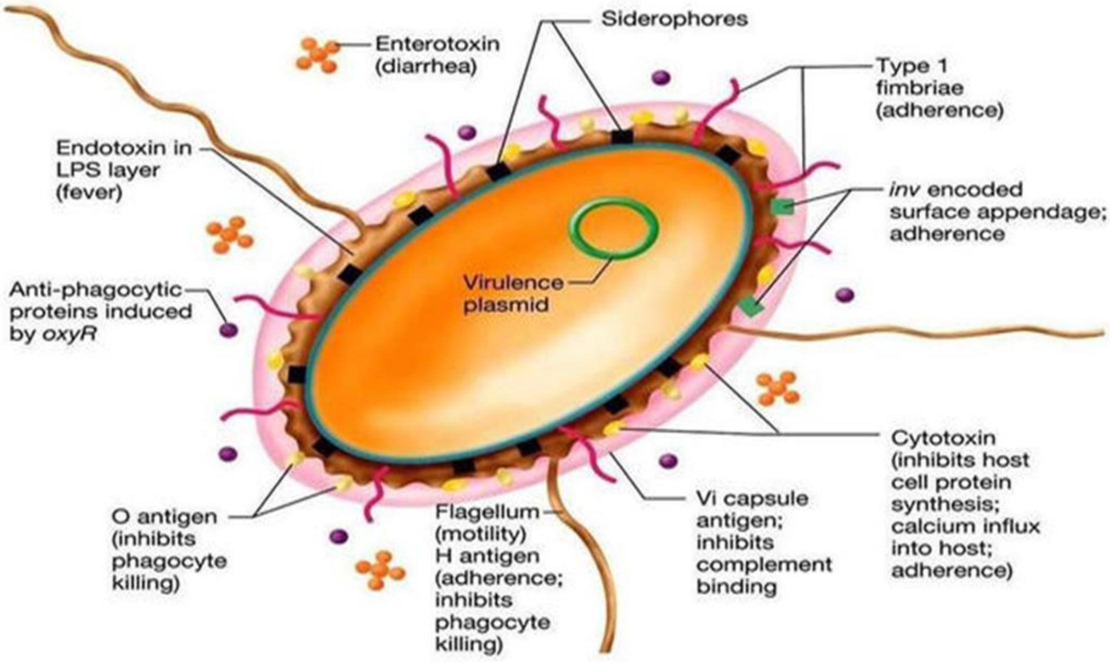

Figure 1: *Salmonella* virulence factors (Al-Khafaji *et al*., 2016)

2.4.5 Pathogenesis of Salmonella

The consequence of *Salmonella* infection depends on agent factors (strains, infective dose), host factors (level of immunity, age), and predisposing factors that influence the host. *Salmonella* enterica is primarily transmitted and infected through feco-oral route. In chickens, for example, *Salmonella* enterica colonizes lower ileum, cecum, and cloaca most selectively and consistently. The ability of *Salmonella* strains to persist in the host cell is crucial for pathogens since *Salmonella* strains lack this ability are non-virulent (Shah *et al.,* 2020).

The digestive tract is the main site of multiplication for *Salmonella*, which may result in widespread contamination of the environment due to bacterial excretion through feces. When the bacteria enter the digestive tract via contaminated water or food, they tend to evade the epithelial cells lining of intestinal mucosa, caeca tonsils, and Payer's patches. The fimbria and flagella present on the surface of *Salmonella* facilitate the initial contact with enterocytes or M cells to colonize the GIT (Foley *et al.*, 2013; Eng *et al.*, 2015).

Once attached to the intestinal epithelia, *Salmonella* expresses T3SS to transport toxins and other effector proteins into intestinal cells. Effector proteins translocate through the Type 3 secretion system induce gastroenteritis, activate secretory pathways, facilitate inflammation, altering ion balances, and consequently, the host develops diarrhea (Ohl and Miller, 2001).

Other important effector proteins facilitate engulfment of the *Salmonella* cells by the host cell membrane, internalized in *Salmonella* containing vacuole (SCVs). Under normal processes, the host cell immune response results in the formation of phagolysosomal fusion to degrade intracellular bacteria. However, SCVs, play an important role in *Salmonella* intracellular survival and proliferation in macrophages by escaping phagolysosomal destruction (Eng *et al.*, 2015; Kabir, 2018).

Inside the macrophages, *Salmonella* expresses SPI-2 T3SSs which are responsible for the secretion of effector proteins in the SCV to project out the bacteria from SCV. Furthermore, SPI-2 T3SSs avoid the fusion of SCV with the lysosomes. Once survive within macrophages and dendritic cells, the organisms reach the main site of multiplication such as the liver and spleen, rich in reticuloendothelial tissue. The survival of *Salmonella* in splenic macrophages is responsible for the slower clearance of this pathogen from the host. The number of bacteria increases in the spleen and migrates to the reproductive tract when birds gain sexual maturity (Foley *et al.*, 2013).

Unlike *S.* Typhimurium, which rapidly causes inflammation of intestinal mucosa and causes Septicemic disease, *S.* Pullorum preferentially targeted the bursa of Fabricius, before eliciting intestinal inflammation and causes enteric disease. Per acute infection and hemolytic anemia both in young and adults chicken resulted from *S.* Gallinarum which are not common in *S.* Pullorum. *S.* Gallinarum is extremely pathogenic to young broiler (Revolledo and Ferreira, 2012; Lee *et al.*, 2015).

2.5. Clinical Signs of Salmonellosis

Overcrowding, transportation, water deprivation, oral antimicrobial medication, and systemic viral and chronic diseases are all stressors that increase clinical Salmonellosis. Increased mortality and poor quality chickens hatched from infected eggs are regarded typical clinical symptoms of fowl typhoid infection. Depression, anorexia, wing drooping, diarrhea, feces adhering to vent, dehydration, anemia, and difficult breathing are among symptoms of older birds (Geetha and Palanivel, 2018; Rahman *et al.*, 2018).

Nonspecific clinical signs like the decline in feed consumption, reduced egg production, and hatchability may be the only signs of *S.* Pullorum. Pullorum disease has the highest mortality rate in 2–3 week old chickens; however the disease may be minor or undetectable in older chickens. Susceptibility is increased at the time of lay in both laying and breeding flocks (OIE, 2012; Eng *et al.*, 2015). The characteristic gross lesions due to salmonella infection include inflammation of body organs; friable, bronze discolored and necrosis of liver; hemorrhagic enteritis congested pneumonic lungs; enlarged and discolored spleen. Unabsorbed yolk and yolk sac infection has been observed during post mortem findings of Pullorum disease in one-week aged chickens (Geetha and Palanivel, 2018).

2.6. Diagnosis of Salmonellosis

Clinical indicators necropsy findings, serological, and molecular diagnostic approaches can all be used to diagnose salmonella infection. *Salmonella* in chicken is diagnosed based on clinical symptoms and pathogen isolation from feces, embryonated eggs, and tissues of affected organs. Isolation and identification of *Salmonella* followed by molecular detection of genus and serotype specific genes using PCR and serotyping of *Salmonella* strains using specific antisera for confirmatory diagnosis (Kabir, 2018; Geetha and Palanivel, 2018).

2.6.1 Bacterial isolation and identification

The “gold standard” for isolation and identification of *Salmonella* is through bacteriological cultural methods. Traditional culture method provided the basis of other supplemental tools to elucidate the causes of microbial disease and to study the clinical and biological features of emerging bacterial diseases. This traditional isolation of *Salmonella* species involves a non-selective pre-enrichment, selective enrichment step, and plating onto selective media. Non selective pre-enrichment medium provides nutrition to recover sub lethally injured *Salmonella* cells while inhibiting the growth of competing flora. Similarly, the application of inhibitors in selective media suppresses the growth of non-*Salmonella* spp. development while allowing continuous growth in *Salmonella* (Lee*et al.,* 2015). According to ISO 6579:2002, buffered peptone water (BPW) is used for pre-enrichment of samples, whereas Rappaport Vassiliadis (RVS) and Muller-Kauffmann Tetrathionate are commonly used for selective enrichment (MKTTn) (ISO, 2002). Biochemical assays, molecular detection, and serological testing can confirm *Salmonella* colonies from selective plating media typically used on XLD and BGA (Houpikian and Raoult, 2002; Ahmed *et al*., 2014).

The conventional culture method serves as the basis for isolation of *Salmonella* due to the ease of use, reliability of results, high sensitivity and specificity, and lower cost compared to molecular based technologies. However, the procedure requires multiple sub culturing tests and hence time consuming compared to molecular diagnostic techniques (Lee *et al*., 2015; Geetha and Palanivel, 2018). Apart from these advantages, *S*. Pullorum and *S*. Gallinarum confirmed by standard culture method rather than serological tests due to their similarity in all features. In this regard, *S*. Gallinarum uses dulcitol, but not *S*. Pullorum, while *S*. Pullorum decarboxylates ornithine, but not *S*. Gallinarum. Recently, chromogenic and fluorogenic growth media such as SM-ID agar, Rambach agar, and BBL CHROMagar *Salmonella* have been used to improve conventional methods. These growth media are used for detection, enumeration, and identification directly from the plate (Perry and Freydière, 2007).

Table 1: Biochemical characteristics of *Salmonella* species

Tests	***Salmonella* Pullorum**	***Salmonella* Gallinarum**	**Other *Salmonella* spp.**
Lactose fermentation	-	-	-
Glucose fermentation	+ (Acid and Gas)	+ (Acid)	+ (Acid and Gas)
Dulcitol	-	+ (Acid)	+ (Acid)
Maltose	± (acid and gas)	+ (Acid)	+ (Acid and Gas)
Indole Production	-	-	-
Methyl red test	+	+	+
Voges-Proskauer test	-	-	-
Motility	-	-	+

Legends + = positive, - = negative

Source: **(**Bishwabidyalay, 2016**)**

2.6.2 Serological diagnosis

Determination of serovars is based on identification of somatic (O) and flagellar (H) antigens using serum agglutination. Immunological assays employ either specific monoclonal or polyclonal antibodies binding with somatic (O), flagella (H), and capsular (Vi) antigens have been mostly used for the detection of *Salmonella* serovars. The Kauffmann-White Scheme is the basis to determine the name of serotype after fully serotyping of the *Salmonella* culture. However, two or more identical serotypes of *Salmonella* can vary with antigenicity due to change and loss of the surface antigens, which reduce the sensitivity of serological methods (Lee *et al.,* 2015; Geetha and Palanivel, 2018).

Agglutination Test

Serogroups can be identified from cultures by antigenic analysis using agglutination reactions subjected to an antigenic structure (Lee *et al*., 2015). Slide agglutination is rapid screening method for identifying both *S*. Pullorum and *S*. Gallinarum under field level. The test prone a significant proportion of false positive results, therefore, tube agglutination test should be employed to screen the sera. Despite chickens can be tested without age difference, some authorities specify a minimum age of 4 months suggested that positive results from chicks, less than 4 weeks may due to maternal antibodies (OIE, 2012).

Enzyme-Linked Immunoabsorbent Assay (ELISA)

Antigens or products of *Salmonella* spp. can be detected by different ELISA systems that have been developed and commercially available kits. In the ELISA assay, an antigen that is specific to *Salmonella* spp. is bound to its specific antiserum and forming an antigen-antibody complex. The concentration of antigen and the presence of *Salmonella* can be quantitatively measured through intensity of color change due to enzyme-substrate reaction. Alternatively, the presence of *Salmonella* antibodies in samples can be detected using antigens coupled to the solid phase of ELISA (Prusak-Sochaczewski and Luong, 1989; Lee *et al*.. 2015).

2.6.3 Molecular detection of Salmonella species

The nucleic acid-based assays in *Salmonella* detection utilize a specific nucleic acid target sequence within organism. DNA amplification (PCR) and direct hybridization (DNA probe) are the two major techniques of molecular methods. These assays allow the detection of very low numbers of organisms in the sample and high throughput of a large number of samples for routine analysis. *Salmonella* invasion gene (invA) is highly conserved among *Salmonella* species and could be serve as a reliable and accurate target gene for molecular detection of the genus *Salmonella* (Guest and Section, 2006; Eng *et al.,* 2015).

The PCR assay is primer meditated enzymatic amplification of specific segments of DNA for detection of pathogens. PCR represents a rapid procedure with high sensitivity and specificity for the immediate detection and identification of specific pathogenic bacteria from different sample types (Ahmed *et al.,* 2014). Apart from its speed, sensitivity, and specificity, the polymerase chain reaction (PCR) is not dependent on the utilization of a substrate and the expression of antigens which may be changed through horizontal gene transfer of plasmid or phage during bacterial evolution (Oliveira *et al*., 2002). The various genus or strain specific as well as virulence associated genes, such as *invA*, 16S rRNA, *agfA*, *viaB*, *hilA* are targeted in PCR assays. Particularly the *invA* gene is a target gene in PCR assays for *Salmonella* detection (Al-iedani *et al*., 2014). Theoretically, the PCR assay can exponentially amplify a single specific sequence of DNA to one million-fold of DNA fragments within 2 or 3 hours using the Taq polymerase enzyme (Guest and Section, 2006; Lee *et al.*, 2015).

2.7. Treatment of Salmonellosis

Clinical cases associated with *Salmonella* infection recover without antimicrobial therapy. Accordingly, therapy should be directed primarily to the replacement of fluid and electrolyte losses due to diarrhea and dehydration. Although the effectiveness of oral fluid therapy may be compromised by malabsorption, oral fluid and electrolyte may be somewhat helpful and much cheaper than IV fluid for animals demanded moderately dehydrated. The prolonged duration of gastrointestinal carrier states and increased relapse of infection result from oral antibiotic therapy that may alter the intestinal microflora (Jemal, 2014).

However, those with severe infections may require effective antibiotic treatment. Application of prophylactic treatment using efficacious drugs for all *Salmonella* serovars may be required to overcoming diversity of *Salmonella* serovars and possession of different virulence factors. The appropriate antibiotic, dose, and length of treatment should be considered since many species of *Salmonella* are prone to developing resistance to many antibiotics (Zhang *et al.*, 2020).

Salmonellosis in human requires early diagnosis and prompt appropriate antibiotic treatment are essential for optimal management, especially in children. Most of the adult cases can be managed at home through fluid therapy, oral antibiotics, and regular follow-up. However, patients with severe illness, frequent vomiting, severe diarrhea, and abdominal distension require hospitalization and parenteral antibiotic treatment. Unlike the newer quinolones and third-generation cephalosporins, which have higher curing rates, standard treatment with amoxicillin or chloramphenicol is associated with relapsing (Andualem, 2014).

2.8. Prevention and Control of Salmonellosis

The reason why prioritization has given in monitoring and control of *Salmonella* infection in the poultry industry is that most human cases of Salmonellosis are thought to be sourced due to consumption of eggs and poultry products (Carrique-Mas and Davies, 2008). Therefore, the objectives of *Salmonella* prevention and control programs are protecting the health of the birds, ensuring safety of the consumers, and strengthing the reliability of poultry production (Ayalew *et al.,* 2017). *Salmonella* prevention and control can be achieved through adopting principles of Hazard Analysis Critical Control Point (HACCP). Strict biosecurity measures, appropriate disinfection, avoiding stressful conditions, and frequent immunization play a significant role in *Salmonella* prevention and control programs. The success in disinfection of chicken farms needs to be tested through taking of samples from different items of poultry environment (Wibisono *et al.,* 2020).

Salmonella Vaccines also help to reduce the amount of *Salmonella* that the animal carries, limit the spread of certain serotypes between farms and into slaughterhouses. However, the use of vaccination as the sole control measure is not recommended. In Ethiopia, an attenuated live vaccine produced from the *S.* Gallinarum 9R strain has been used. Day-old vaccination against *Salmonella s*erotypes should be considered when the health status of the breeder flock or the hatchery from where the flock originates is not known. when moving day-old birds to contaminated farms, vaccination against the *Salmonella* serotypes is known to minimize the risk of salmonella infection (Crouch *et al*., 2020).

Salmonella infections due to non-typhoidal *Salmonella* species are the major public health problem worldwide. Therefore, in humans, prevention and control of Salmonellosis is mainly depending on proper cooking, adequate refrigeration, and avoiding eating of raw foods of animal origin to limit entrance of zoonotic *Salmonella* into the human food chain. Wider use of pasteurized eggs in restaurants, hospitals, and nursing homes are also important prevention measure. Education and public awareness of food industry workers in basic food safety and restaurant inspection procedures may prevent cross-contamination. Poultry vaccination will more attractive since other available methods are not costs effective, difficult to implement, and increasing problems associated with antibiotic resistance (Crump *et al.,* 2015; Ayalew *et al.*, 2017).

2.9. Antimicrobial Resistance of *Salmonella* Species

Resistance to the most commonly used antimicrobials agents in animal production systems is a critical concern in *Salmonella* strains (Uddhav and Sivagurunathan, 2016). The incidence of antibiotic-resistant *Salmonella* strains to commonly used antibiotics in humans is originating from food animals. A single antibiotic resistant (chloramphenicol), *Salmonella* was reported in the early 1960s for the first time. Since then, the isolation of single or multidrug-resistant *Salmonella* serotypes has been more common. In recent years, several clones of multidrug-resistant (MDR) *Salmonella* have emerged, and their prevalence in humans and domestic animals species has expanded globally (Adesiji *et al*., 2014; Jajere, 2019). Previous studies on antibiogram profiles of *Salmonella* spp. revealed that most serovars of *Salmonella* were resistant to ampicillin, amoxicillin, tetracycline, oxytetracycline, and streptomycin in humans and animals (Garedew *et al*., 2015; Tadesse, 2015; Dagnew *et al*., 2020).

Genotypic analysis of antimicrobial resistance showed that one or more genes are responsible for the development of antimicrobial-resistant *Salmonella* strains. For example, the genes like *tetA*, *sul1*, *cat1*, *aph* have been known responsible genes for the development of resistance to tetracyclines, sulfonamides, chloramphenicol, and aminoglycosides, respectively (Adesiji *et al.*, 2014). Genes that are responsible for antimicrobial resistance property of *Salmonella* against common antibiotics are located on mobile genetic elements (plasmids and variants of *Salmonella* Genomic Islands SGI1 and SGI2) which transfer horizontally (Eng *et al*., 2015; Carroll *et al.*, 2017).

Microorganisms become resistant to antimicrobials in one of two ways: Either intrinsically, whereby some microorganisms lacking the target sites for specific antimicrobials or naturally susceptible microorganisms acquire resistance ways that enables them not to affected by antimicrobials. Bacteria have evolved several and sophisticated antimirobial resistance mechanisms, including reduction in entry to the target site, activation of efflux pumping to expel antimicrobial against from the cell, alteration, amplifying and protecting antimicrobial drug targets and enzymatic destruction of antimicrobials to reduce efficacy (Brötz-Oesterhelt and Brunner, 2008; Davies and Davies, 2010; Kumar and Singh, 2013).

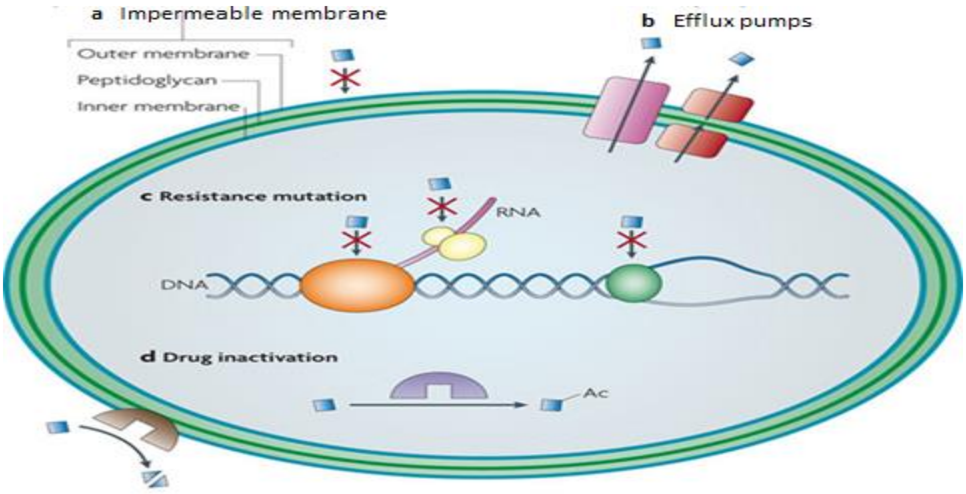

Figure 2: Mechanisms of drug resistance in a Gram negative bacterium (Allen *et al.*, 2010).

There are three antimicrobial susceptibility testing methods namely, disk diffusion, broth dilution, and agar dilution that provide reproducible results when followed correctly. Broth and agar dilution methods are the most commonly used techniques to determine the minimal inhibitory concentration (MIC) of antimicrobial agents that inhibit the growth of bacteria (Wiegand *et al.,* 2008). Disk diffusion, also known as Kirby Bauer disk method, is a simple and practical method that uses a defined concentration of antibiotic impregnated disk to test susceptibility of a particular bacterial species to specific antimicrobial agents (Uddhav and Sivagurunathan, 2016). Antimicrobial agents diffuse from disks into the solid culture medium (Mueller Hinton agar medium) which has seeded with pure bacterial isolates. If the organisms are inhibited by defined concentration of antibiotics, zone of growth inhibition will be formed immediate area around the disks. The result of the disk diffusion method can be determined based on zone of inhibition and directly proportional to concentration of antimicrobial agent and bacterial susceptibility to that antimicrobial agent (Cockerill *et al*., 2012; Eng *et al.*, 2015; Mensah *et al*., 2019).

The extensive use or misuse of the antimicrobial agents (therapeutic, prophylaxis, and growth promotant), use of antibiotics under dose level, over prescription at health facilities has led to the emergence of multidrug-resistant (MDR) bacteria. Most bacteria acquired resistance in the animal before being transferred to humans through the food chain. Therefore, the public health risk of Salmonellosis is due to the transfer of resistant strains to humans through the consumption of contaminated food of animal origin (Geresu *et al*., 2021).

2.10. Economic and Public Health Importance of Salmonellosis

Salmonella infection has significant consequences in domestic chickens both in developed and developing nations of the world (Kassaye *et al.,* 2015). The economic impacts of this disease include productivity losses for the poultry farms (production losses, death in any age group of chicken, increased culling rate), cost of treatment, prevention and control, market disturbances (Nair *et al.*, 2015; Jajere, 2019).

None typhoidal *Salmonella* serovars have a public health concern since most strains of *Salmonella* are potentially pathogenic to humans and animals (Nair *et al.*, 2015*)*. Serovars typhimurium, and more recently enteritidis have been most frequently isolated from cases of human food poisoning in which chicken products have been implicated. Recently *Salmonella* infection in humans has been increased as the result of the frequent occurrence of animal salmonellosis (Kabir, 2018; Ayalew *et al.*, 2017). This is, therefore, animal salmonellosis is considered the principal reservoir and source for the occurrence of human salmonellosis (Junior *et al*., 2010; Tadesse and Gebremedhin, 2015; Gebremedhin *et al.*, 2021).

Infection as the result of resistant *Salmonella* strains has also significant clinical relevance as it can increase the morbidity, mortality, and costs associated with the disease. This infection requires life-threatening interventions with third-generation antibiotics like quinolones and cephalosporin, cost large amounts of money (Castro-Vargas *et al.,* 2020). The occurrence of multidrug-resistant *Salmonella* also resulted in increased severity of diseases and prolonged syndromes both in humans and animals. Antimicrobial-resistant *Salmonella* in poultry and other production animals is a potential risk for the dissemination of antimicrobial resistance to humans (Kagirita *et al*., 2017).

2.11. The Status of Salmonella Infection in Ethiopia

2.11.1 Prevalence

Salmonella infections have been recognized almost in all countries, but appear to be more prevalent in areas where intensive poultry, cattle, and swine farming (Nair *et al.*, 2015). Previous studies carried out on animals, food of animal origins, animal environments, and humans in Ethiopia has revealed that *Salmonella* species are quite prevalent (Kassaye *et al.*, 2015). Salmonella infections have been isolated in both broiler and layer breeds and various species. However, the estimates of prevalence in commercial poultry farms have varied. A summarized compilation of representative previous studies were presented in Table 2.

Table 2: Prevalence of *Salmonella* in Ethiopia in different animal species

Area	**Sources of samples**	**Prevalence**	**Reference**
Adama and Mojo	Poultry	2.9%	(Dagnew *et al*., 2020)
Debire Markos	Food handlers	3.6%	(Mengist *et al.*, 2018)
Jimma	Poultry (eggs)	2.65%	(Taddese *et al*., 2019)
Haramaya	Poultry (eggs)	2.9%	(Tessema *et al*., 2017)
Bishoftu	Cattle and human	3.77%	(Merera, 2018)
Arsi Zone	Food items	9.4%	(Geresu *et al.*, 2021)
Gondar	Cattle	17.3%	(Garedew *et al.,* 2015)
Assella	Cattle	6.50%	(Beyene *et al.*, 2016)
Ambo and Holeta	Cattle	5.7%	(Gebremedhin *et al*., 2021b)
Mojo	Poultry	19.41%	(Abuna *et al.,* 2016)
Meki Town	Dairy farms	11.2%	(Abunna *et al.*, 2018)
Haramaya	Poultry	2.7%	(Kemal *et al.*, 2016)
Holeta	Cattle	5.60%	(Abuna *et al*., 2017)
Southern Ethiopia	Poultry	16.67%	(Abdi *et al.*, 2017)
Dessie	Cattle	4.95%	(Amera *et al.*, 2017)
Eastern Hararghe	Cattle and shoat	5.05%	(Kassa *et al.*, 2017)
In and around AA	Poultry	14.6%	(Eguale, 2018)
Holeta	Dogs	17.1%	(Sultan *et al.*, 2018)
Bishoftu and Mojo	Poultry	14.6%	(Asfaw *et al*., 2020)

In the prevalence of *Salmonella*, true differences have been identified across geographic regions, management systems, detection techniques, sample types, collection, and handling methods (Ayalew *et al.,* 2017; Asfaw *et al.,* 2020). *Salmonella* serovars frequently reported in Ethiopia include *S.* Typhimurium, *S.* Newport, *S.* Saintpaul, *S.* Kentucky, *S.* Haifa, *S.* Enteritidis, *S.* Gallinarum, *S.* Anatum, and *S.* Pullorum (Aragaw *et al.,* 2010; Eguale, 2018; Ketema *et al.,* 2018; Dagnew *et al.,* 2020).

2.11.2 Antimicrobial resistance

Antimicrobial agents have a distinct role in the prevention and control of various infectious diseases, promotion of growth, and prophylactic treatment. However, indiscriminate and non-judicious extensive use of drugs might lead to the emergence of multidrug-resistant strains of *Salmonella* (Waghamare *et al., 2*018). During the past two decades, antibiotic-resistant *Salmonella* has emerged and become a serious public health issue worldwide. Studies conducted previously in Ethiopia indicated that the presence of a high level of single or multi drug resistance in *Salmonella* isolated from chicken-related samples (Abunna *et al.*, 2016; Eguale, 2018), fecal samples taken from dairy cattle (Eguale *et al*., 2016), food animals and food products (Ferede, 2014; Kebede *et al*., 2016; Geresu *et al*., 2021). Antimicrobial resistance has also been detected in *Salmonella* isolated from humans (Andualem, 2014; Merera, 2018).

Many research findings have been reported that almost all isolates of *Salmonella* are resistant to one or more commonly used antimicrobials agents especially ampicillin, oxytetracycline, and tetracycline. However, several disc-diffusion antimicrobial susceptibility tests showed that none or a limited number of *Salmonella* isolates are resistant to ciprofloxacin and other quinolones (Ferede, 2014; Merera, 2018). Strainwise antimicrobial resistance showed that *Salmonella* strains such as *S*. Kentucky, *S*. Typhimurium, *S*. Concord, and *S*. Saintpaul have been found resistant to many antibiotics (MDR). Surprisingly, *S*. Kentucky strains have been found resistant to ciprofloxacin, despite the fact that all isolates were susceptible in earlier studies. However, still, there hasn’t been well-documented information about genes that are responsible for the development of antimicrobial resistance in most *Salmonella* serovars (Eguale *et al*., 2015; Eguale, 2018; Merera, 2018). Previous research findings elsewhere in Ethiopia showed that several factors contribute to the emergence of antibiotic resistance in Ethiopia (Kebede *et al.,* 2016b; Asfaw *et al*., 2020; Geresu *et al*., 2021).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in and around Debire Birhan, a town located 130 kilometers North-East of Addis Ababa. Debire Birhan is the administrative center of Amhara Regional State's North Shoa Zone and is located at 9°41′ N 39°32′ E co-ordinates, with an elevation of 2700 to 2800 meters above sea level (masl). The climate condition is characterized by a bimodal rainfall pattern consisting of a long rainy season (June to September) and a short rainy season (February to May) and an extended dry season from late November to February. The annual rainfall and humidity are 971.36 mm and 62.7%, respectively. The average temperature record is 10^{o}C. The surrounding areas namely Deneba, chacha, and keyit are the administrative center for Siyadebirna Wayu, Angolelana Tara, and Basona Werana districts respectively. These areas have comparable climatic and weather condition to Debire Birhan town except Deneba which has relatively warmer weather conditions and 45 km far from it (North Shewa Zone Office of Agriculture and Rural Development, 2013).

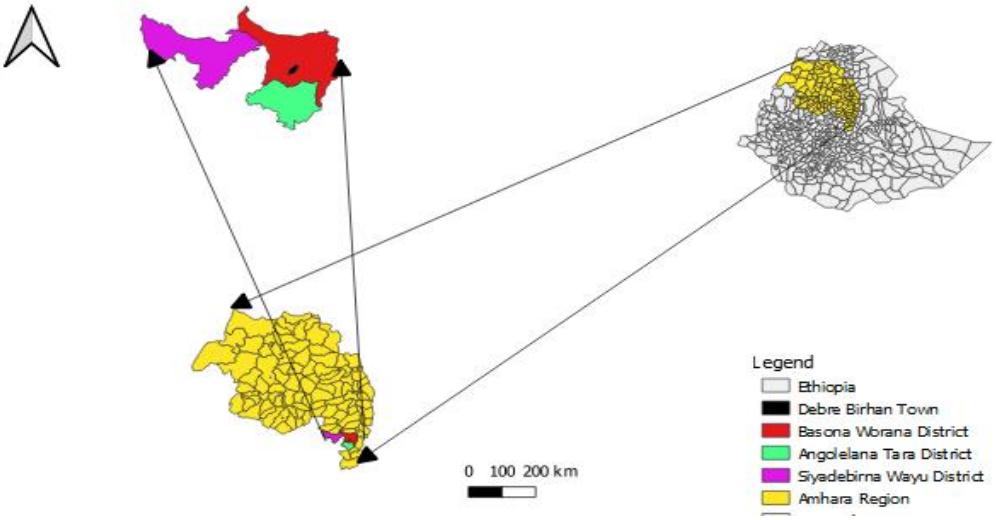

Figure 3: Map representing the study area where samples were collected using QGIS Ver. 3.14.

In the area, agricultural activities such as mixed farming, livestock production (poultry, dairy, and small ruminant fattening) with certain industrial manufacturing activities were the main source of income. Nowadays, among the livestock production sectors, poultry production has been fast expanding and a major income generating sector. According to CSA (2019/20) the total number of chickens in North Shoa, Amhara Regional State is 1,578,906. In the area, few numbers of native and non discriminatory types of chickens were accessible as compared to exotic chickens (Bovans Brown and Saso breeds). like the other central parts of the country, there has been a progressive development in market oriented small and medium scale poultry production in Debire Birhan due to the efforts of the Ethiopian government to increase the productive potential of domestic birds and creation of job opportunities by introducing and disseminating exotic breeds mainly Bovans Brown and Saso (North Shewa Zone Office of Agriculture and Rural Development, 2020).

3.2. Study Animals and Study Design

A cross-sectional study design was employed from December 2020 to June 2021 to perform isolation, molecular detection, antibiogram profile, and to assess the associated risk factors of *Salmonella* species from samples collected aseptically from selected poultry farms in and around Debire Birhan, Central Ethiopia. The study animals comprised apparently healthy exotic Bovans and Saso breed chicken with different age groups, flock sizes and maintained under an intensive management system. However, apparently healthy chickens that had a history of fowl typhoid vaccination were purposively excluded from this study. Representative poultry farms in Debire Birhan town and three other towns at the outskirt of Debire Birhan purposively selected based on the data obtained from the livestock and fishery office of North Shoa Zone, Amhara Regional State. The area selection was made referring the high chicken population and accessibility to transportation.

3.3. Sample Size Determination and Sampling Strategies

The appropriate sample size for this study was calculated taking into account 50% expected prevalence, 95% confidence interval and 5% absolute precetion as there was no previous study conducted on phenotypic and/or molecular characterization as well as antibiogram profile of *Salmonella* species from poultry and their environments in the study area. As per the formula given by Thursfield, the sample size was found to be 384 (Thrusfield, 2007).

$$N = \frac{(Z_{\alpha/2})^2 \times P(1 - P)}{d^2}$$

Where

N = Sample size required

d = absolute precession (0.05)

p = expected prevalence

It was noted that there were 40, 20, 10, and 10 poultry farms in Debire Berehan, Deneba, Keyit, and Chacha, respectively. From the aforementioned four areas, a total of 26 farms (about 30% of the total farms in each area) were purposively selected based on the number of poultry farms. Accordingly, 13, 6, 4 and 3 poultry farms were selected from Debre Birhan town, Deneba, Keyit and Chacha, respectively. The 384 samples were obtained from purposively selected 26 poultry farms out of the above listed number of farms located in and around Debire Berehan area. The selection of farms in each area for sampling was based on the willingness of farm owners, age, and the number of chickens. Based on the flock size, farms were categorized into small (50-500), medium (500-1500), and large (≥1500) birds per farm (Sharma *et al.*, 2021). Similarly, chickens were also categorized into different age groups as rearing (< 8 weeks), pullets (9-18 weeks), and laying hens (≥ 18 weeks).

The number of samples in each poultry farm was determined by taking 3 to 5% of the total flock size considering the flock size and the number of feeders and waterers present in each chicken farm (Dagnew *et al*., 2020). The total number of samples obtained from a given farm were proportionate to each sample type. Samples, particularly swab and fecal samples, were pooled together (at least from three chickens and fecal droppings). The required feeders and waterers were randomly selected from each poultry house. Therefore, out of the total samples size (n=384) from poultry and different poultry items were taken and thus 192, 84, 62 and 46 were obtained from Debre Birhan, Deneba, Keyit, and Chacha, respectively. Each selected chicken farm was sampled just once during the study period.

3.4. Sample Collection, Transportation and Storage Techniques

Samples were collected according to the recommendations of OIE (2012). 25 gram pooled fresh fecal droppings (at least from three droppings) from each poultry house were collected with a sterile spatula into the sterile universal bottle. Pooled cloacal swabs (at least from three chickens) were collected from randomly selected chickens using sterile cotton tipped swabs. Cotton swabs were moistened in buffered peptone water solution before being inserted in the cloaca and by gentle rotation in the cloaca of birds. Immediately the cloacal swabs were kept in 10 ml glass tubes containing sterile buffered peptone water, properly plugged and shaken within test tubes for uniform distribution of microorganisms. Furthermore, 25 gram pooled feed samples from randomly selected chicken feeders were collected aseptically and kept in a sterile universal sampling plastic bottle. Similarly, at least 100 ml pooled water samples from chicken drinking water were collected into sterile plastic cups. All samples were properly labeled with a permanent marker to identify the date of sampling, site of the farm, age, breed, and flock size of the farm.

Upon completion, samples were kept in an ice-box containing ice packs and immediately transported to Veterinary Microbiology Laboratory of the College of Veterinary Medicine and Agriculture of Addis Ababa University. Finally, the collected samples were processed upon arrival or kept in the refrigerator overnight. Due attentions were taken during sample collection, transportation, and laboratory processing to avoid cross contamination (Djeffal *et al.,* 2018a).

3.5. Isolation and Identification of the *Salmonella* species

Standard bacteriological analyses were performed according to the protocol International Organization for Standardization for *Salmonella* detection in food and animal feedstuffs, ISO 6579:2002, and OIE Terrestrial Manual (2012). According to the standard, Buffered Peptone Water (BPW) (Himedia, India) and Rappaport Vassiliadis (RVS) (Himedia, India) were used as a non-selective pre-enrichment and selective enrichment media, respectively. Xylose Lysine Deoxycholate Agar (XLD) (Newmubia, India) was used as selective plating media. The bacteriological solid or broth media used for the isolation and identification of the genus *Salmonella* were prepared according to the instructions of the manufacturer (Djeffal *et al.*, 2018a).

Non selective pre-enrichment

In case of overnight frozen samples, thawing for 3 to 5 hours was done prior to processing. Following thawing, 25 grams of pooled fecal and feed samples were pre-enriched with 225ml of BPW (1:9) proportion and homogenized by massaging for two minutes. Each 100 ml of drinking water sample was also mixed with 100ml of BPW (Annex 4A). Cloacal swabs were soaked in 10ml BPW during sample collection and all pre enriched samples were incubated at 37°C for 18-24 hrs (Daquigan *et al.*, 2016; Djeffal *et al.*, 2018b).

Selective enrichment

Following non-selective pre-enrichment, 0.1ml (100μl) of cultured broth was inoculated aseptically into tubes containing 10ml of RV broth after vortexing. Inoculated RV media were mixed well and incubated at 42^0C for 18-24 hours (OIE, 2012).

Plating on Selective media

Selective plating media, Xylose lysine deoxycholate (XLD) was prepared according to the manufacturer recommendation (Annex 1a). Prepared culture media were kept in a sterile refrigerator if immediate inoculation could not possible and were thawed for 3 to 5 hours at room temperature before inoculation. Following incubation, a loopful of suspension from the edge of the turbid growth zone of each culture was streaked onto XLD and incubated at 37°C for 24 to 48 hours. Next to incubation, plating Media were examined for the presence of typical *Salmonella* colonies, and then presumptive *Salmonella* colonies were sub cultured on the same plating media to get pure colonies. However, plates were incubated for further 24 houres and reexamined if typical *Salmonella* colonies were not present. Salmonella colonies on XLD were identified by the formation of red colonies with black centers (Annex 4B). Then, typical *Salmonella* colonies were confirmed through six biochemical tests explained below (Busse M., 1995; Park *et al.,* 2012).

Triple Sugar Iron Agar (TSI) Test

Triple sugar iron agar media were prepared based on the instruction of the manufacturer (Annex 1e). Following media preparation, typical *Salmonella* colonies from subcultured plating media were selected and streaked onto the nutrient agar plates (CM0003, England) and incubated at 37^0C for 18 to 24 hours. Following refreshing of the *Salmonella* suspected colonies, TSI agar slant tubes were inoculated by a loopful of *Salmonella* colonies from the nutrient agar plate using stab streak method and incubated at 37^0C for 24 hours. After incubation, the growth of *Salmonella* was identified by the formation of alkaline slant, yellow butt, and black precipitation due to the production of hydrogen sulfide (H_2S) (Annex 4C) (Islam *et al.*, 2018).

Citrate Utilization Test

Following media preparation (Annex 1f), Simon’s citrate agar (Himedia, India) tubes were inoculated by streaking the surface of the slant with a loopful of fresh *Salmonella* colonies from the nutrient agar. After loosely capping, inoculated tubes were incubated aerobically at 35 to 37°C for up to 4 days. Finally, a positive test was demonstrated by a color change from green to intense blue along the slant (Annex 4D) (Haque and Sao, 2015).

Indole Test

The Tryptone Soya Broth (TSB) media (Oxoid, England) was prepared based on the manufacturer's instructions (Annex 1h). Two to four ml TSB broth media were inoculated with 18 to 24 hours cultured fresh *Salmonella* colonies from the nutrient agar plates and incubated at 37^0C for 18 to 24 hours. Next to this, 0.5 ml Kovac's reagent was added to each broth culture. Finally, the absence of red ring formation was interpreted as *Salmonella* positive result (Annex 4H) (Macwilliams, 2009; Haque and Sao, 2015).

Methyl-Red and Voges-Proskauer (MR-VP) Test

After preparation of MR-VP broth media (Newmubia, India), about two ml was inoculated with fresh colonies of *Salmonella* from the nutrient agar plate and incubated at 37 ^{0}C for a minimum of 48 hours. Five drops of methyl red solution for methyl red test; six and four drops of Alphanaphthanol and potassium hydroxide solution, respectively for VP test were added to incubated broth culture (Annex 4H). *Salmonella* positive results were interpreted using appearance of bright red color immediately after adding of methyl red solution and absence of red-brown color formation at the surface of the tube cap within 30 min of adding VP reagents and properly shaking (Annex 4E/G) (Bishwabidyalay, 2013; Mcdevitt, 2016).

Lysine Decarboxylation Test

Sterilized lysine iron Agar broth medium was inoculated with one or two pure *Salmonella* colonies from the nutrient agar plate, cultured for 18 to 24 hours. Following inoculation, tubes were overplayed by 1 ml paraffin oil (up to 4 mm), tightly capped, and incubated at 37 ^{0}C for 18 to 24 hours, and observed daily for up to 7 days. *Salmonella* positive results were interpreted by the appearance of turbid purple due to lysine decarboxylation (Annex 4F) (Mcdevitt, 2016).

Maintenance of Stock Culture for *Salmonella* Isolates

Bacterial colonies from biochemically confirmed isolates were subcultured to XLD plating media. After incubating subcultured plating media for 18 to 24hrs, typical *Salmonella* colonies were inoculated into prepared TSB and incubated for 18 to 24 hrs at 37 ^{0}C. Following incubation, Salmonella broth culture was overlayed by paraffin oil and stored at +4 refrigerator. These *Salmonella* broth cultures were subcultured every two weeks interval adhering to similar procedures until molecular analysis was done (Islam *et al.*, 2018).

3.6. Molecular Detection of *Salmonella Typhimurium*

Molecular detection of *S.* Typhimurium was conducted at Molecular Laboratory, National Veterinary Institute (NVI), Bishoftu, Ethiopia. Of the total biochemically verified *Salmonella* isolates, molecular detection of Spy gene (specific to *S.* Typhimurium), was employed for 30 purposively selected isolates to incorporate all the risk factors as mutch as possible.

3.6.1 DNA extraction

Extraction of DNA was carried out from frozen stocks of *Salmonella* broth culture recovered in TSB using DNeasy Blood and Tissue kit extraction kit (Qiagen, Germany) as per the instruction provided by the manufacturer. The frozen *Salmonella* isolates in TSB broth and other required reagents were thawed at room temperature before processing. 200 µl aliquots of specimens were mixed with 200µl Lysis buffer (AL) in a 1.5ml microcentrifuge tube for lysing the cell. The suspension was vortexed for 15 seconds to mix thoroughly for efficient lysis of the cell. Then the homogenized solution was incubated at 56^0C for 10 minutes followed by centrifugation to remove any drops from the inside of the lid. Ethanol (97%) (200 µl) was added to each sample, for better DNA precipitation, mix by vortexing and briefly centrifuged to remove drops from the inside of the lid. The mixture was carefully allocated to QIAamp spin column tubes (Hilder, Germany). The spin columns were immediately capped and centrifuged at 6000xg (8000 rpm) for one minute. The QIAamp spin columns tubes were placed in clean collection tubes (2 ml) and tubes containing filtrate were discarded. 500 µL washing buffer 1 (AW1) was added into spin column tubes and centrifuged with the same speed and time. Following discarding the filtrate, 500 µl washing buffer 2 (AW2) was added into spin column tubes and centrifuged with full speed (20000xg; 14000rpm) for three minutes. Then, QIAamp spin Columns tubes were placed in clean 1.5ml microcentrifuge tubes and collection tubes containing filtrate were discarded. Elution buffer (200 µl) (AE) was added to spin column tubes and incubated at room temperature for 5 minutes. Finally, incubated QIAamp spin column tubes containing AE buffer were centrifuged at 6000xg (800 rpm) for one minute. Finally, pure DNA templet was stored at (-20^0C) until gel electrophoresis has done.

3.6.2. Conventional polymerase chain reaction (PCR)

Master Mix preparation and DNA amplification

The Polymerase Chain Reaction (PCR) was conducted using a thermal cycler (Applied Bio-systems) for amplification of *S.* Typhimurium specific gene (Spy). The forward and reverse primers set for this gene are shown in table 3. The PCR reaction was standardized in a final volume of 20µl containing; nuclease free water (3µl), 5 pmol/µl each forward and reverse primers (2µl for each), I Q ^{TM} supper mix (10µl) (BIO-RAD, USA) containing (Taq DNA polymerase, dNTPs, $MgCl_2$ and PCR buffer) and DNA templet (3µl). Likewise, positive control (*Salmonella* positive templet), extraction control (devoid of template DNA) and negative control (nuclease-free water) were also prepared (Table 3).

DNA amplification was carried out following reaction conditions: an initial denaturation step at 95^{0}C for 5 minutes; followed by 35 cycles of denaturation at 95^{0}C for 30 seconds, annealing at 52^{0}C for 40 second, and extension at 72^{0}C for 30 second with 7 minutes final extension at 72^{0}C and holding temperature at 4^{0}C until analysis.

Table 3: Reagents and primers used for master mix preparation

No.	**Type of reagent**	**For one reaction**	**Total 30 reactions**
1	RNase free water	3 µl	90 µl
2	Primer – Spy-Fow-5pm/µl 5’- TTA TTC ACT TTT TAC CCC TGA A- 3’	2 µl	60 µl
3	Primer – Spy-Rev-5pm/µl 5’- CCC TGA CAG CCG TTA GAT ATT- 3’	2 µl	60 µl
4	I Q TM Supermix	10 µl	300 µl
5	Template	3 µl	90 µl
	Total	20 µl	600 µl

Gelectrophorosis DNA band visualization

After amplification, the PCR fragments were checked using agarose gel electrophoresis and visualized using UV light. Before gel electrophoresis, agarose gel (1.5%), loading dyes, and molecular marker (100-bp) were prepared based on the manufacturer's recommendation (Annex 3a). The total volume of 10 µl mixture of PCR products and loading dye were loaded on 1.5% agarose gel wells which were prepared from 1% TAE Buffer (Tris-acetate-EDTA) and agarose powder. Similarly, extraction, positive and negative controls (10 µl each) and molecular ladders (10µl, 100 bp) were gently loaded on the separate agarose gel wells (lane). Consequently, the amplified DNA product was electrophoresed at 120 volts for one hour. The migration of DNA bands from the agarose gel was visualized using UV gel documentation apparatus.

The amplicons (bands) size of around 401 bp of target gene (Spy) for *S.* Typhimurium was visualized and captured on a UV transluminator. The presence of visible bands at or around the expected size (401) was considered positive whereas the absence of bands at the expected size was considered negative. Finally, the bands were saved, printed out, and documented for reporting (Malorny *et al.*, 2003; Shanmugasamy *et al.*, 2011; Xiong *et al.*, 2018).

3.7. Antimicrobial Susceptibility Test

The Disc-diffusion method was employed for antimicrobial susceptibility testing of 15 PCR confirmed *S.* Typhimurium isolates. This was based on the recommendations of the National Committee for Clinical Laboratory Standards (CLSI) on Muller Hinton agar medium. Before implementing disc-diffusion method, a specialized media for antimicrobial sensitivity testing, Muller Hinton Agar Medium, was prepared based on the standard described by (CLSI, 2012).

Fresh *Salmonella* colonies were grown on a nutrient agar plate from each PCR confirmed *S.* Typhimurium broth culture stored in the refrigerator. Four to five colonies were transferred with the sterile loop into tubes containing 4ml of saline water and incubated at 37°C for 6 hours until it achieved 0.5 McFarland turbidity standards (Annex 2b). This standard was adjusted by adding a sterile saline solution or isolated colonies from the nutrient agar. After dipping and rotating in the suspension, the swabs were pressed firmly on the inside wall of the tubes above the fluid level to remove excess inoculums. The smooth suspension was inoculated uniformly on the entire surface of Mueller-Hinton agar (Oxoid, England) (Annex 2a) by using sterile cotton swabs and inoculated plates were held at room temperature for 3 to 5 minutes to allow air drying.

Antimicrobial drugs were chosen based on their widespread usage in the treatment and/or prevention of *Salmonella* infection in livestock production and human health, as well as their accessibility in local markets. Therefore, 10 antimicrobials namely tetracycline (TET 30 µg), oxytetracycline (OT: 30 µg), ampicillin (AMP: 10 µg), sulfamethoxazole (SXT: 25 µg), gentamicin (GEN: 10 µg), kanamycin (KNA: 30 µg), erythromycin (ERT: 15 µg) nalidixic acid (NA: 30 µg), chloramphenicol (CHL: 30 µg) and ciprofloxacin (CIP: 5 µg) were tested for 15 *S*. Typhimurium isolates.

Antibiotic disks from each selected antibiotic were placed on the Muller Hinton Agar plate at least 15mm apart using sterile forceps to avoid overlapping of the inhibition zone. The plates were incubated at 37^0C for 24 hrs. The diameter of clear zone of inhibition was measured into the nearest mm by using a caliper, which is held on the back of the Petri dish (Annex 4J). The results of antimicrobial sensitivity test were interpreted as sensitive, intermediate, or resistant according to the interpretation cut-off points for susceptibility status of bacterial isolates (CLSI, 2013) (Annex 6).

3.8. Data Management and Analysis

The raw data were entered and managed in a Microsoft Excel worksheet and imported to R software version 4.10 computer programs for further data analyses. The isolation and detection rate of *Salmonella* spp. was calculated for all data by dividing *Salmonella* positive samples by the total number of observations and multiplied by a hundred. The association between the possible risk factors (site, age, breed, source of sample, and flock size) and isolation rate of *Salmonella* species using the conventional culture method was analyzed by Chi-square (χ^2). However the association between explanatory variables and the detection rate of *S*. Typhimurium using PCR was computed by fisheries exact test since some of the variables contained expected frequency less than or equal to 5. Finally, in both cases, the association of the variables and the isolation and detection rate were considered statistically significant if the computed *p*-value is less than 0.05.

3.9. Ethical Clearance

This research is parts of AAU funded thematic project owned by Hika Waktole (Assistant Professore). Ethical clearance for the study was given by the Animal research ethical review Committee of Addis Ababa University, College of Veterinary Medicine and Agriculture in a letter dated on 21/04/2021 and reference number VM/ERC/04/13/021. The significance of this research was evaluated from ethical perspectives, applicability, and originality technical competence point of view.

Limitations and Constraints of the Study

The current finding differed from prior research in terms of phenotypic characterization and molecular detection rate of *Salmonella* isolates for a variety of reasons. In terms of the sample, most of the chicken farms in the study area were reared layer chickens. It was not possible to conduct post-mortem as chickens were belonging to the farms. Although this study was initially proposed to detect and molecularly characterized the *Salmonella* species, due to a lack of serotype-specific primers, especially for chicken host-adapted *Salmonella* serotypes like *S.* Gallinarum and *S.* Pullorum, and reference strains, culturally isolated *Salmonella* species were not molecularly described deeply.

As the result of an expensive cost and lack of availability of antimicrobial discs to purchase the required amount from the local market, only a small number of *Salmonella* isolates were tested for limited antimicrobial agents in the current investigation. Similarly, very indicative plating media for *Salmonella* isolation (Brilliant Green Agar) and media for confirmatory biochemical tests (Urease test) were exclude from this study due to the reasons mentioned above. The declaration of a state of emergency due to the COVID-19 epidemic, the country's instability for virtually the whole year, and other challenges were greatly contribute to the delay in not being able to present in the first round.

4. RESULTS

4.1. Isolation and Identification of *Salmonella* Species

Bacteriological analysis of 384 samples collected from cloacal swab (n=136), fecal droppings (n=130), chicken feed (n=64), and chicken drinking water (n=54) revealed that 14.06% (54/384) samples were confirmed the presence of the genus *Salmonella*. Of the total of 26 poultry farms examined in the present study, the *Salmonella* spp. were isolated from 73.07% (19/26) (Table 4). *Salmonella* species were not isolated from seven poultry farms (3 farms from Deneba, 2 farms each from Chacha and Debire Birhan). The higher level of isolates were recorded from farms in Keyit (100%) followed by Debire Birhan (84.61%) and Deneba (50%), while the lower isolation rate was observed in Chacha (33.33%).

Based on the bacteriological method, the isolation rate of *Salmonella* spp. was: Debire Birhan (15.62%), Deneba (10.71%), Keyit (19.35%), and Chacha (6.52%) (Table 4). This study also revealed that sample level isolation and identification rate of *Salmonella* spp. was relatively higher in Keyit (19.35) followed by Debire Birhan (15.62%) and Deneba (10.71) (Table 4).

Based on the standard bacteriological culture methods, the rate of isolation and identification of rate of *Salmonella* spp. was13.23%, 22.30%, 6.25%, and 5.55% in cloacal swab, fecal sample, feed, and water samples, respectively (Table 5). Thus, the higher percentage of *Salmonella* spp. was isolated from fecal samples (22.30%).

Table 4: The isolation rate of *Salmonella* species based on bacteriological culture methods.

Study sites	**No. of Farms**	**No. of sample**	**No. of Positive farms**	**No. of positive samples**	**Positive farms (%)**	**Positive samples (%)**
D/Birhan	13	192	11	30	84.61	15.62
Deneba	6	84	3	9	50.00	10.71
Keyit	4	62	4	12	100	19.35
Chacha	3	46	1	3	33.33	6.52
Total	26	384	19	54	73.07	14.06

Besides, the percentage of occurrence of *Salmonella* spp. showed variability in terms of flock sizes. *Salmonella* species were more commonly identified in poultry farms with higher flock sizes (23.25%) as compared to medium (13.91%) and small sized (6.73%) flocks of greater than or equal to 1500 chickens than flock sizes 50-500 and between 500 and 1500 chickens (Table 5). This finding also showed that the isolation rate of *Salmonella* spp. across breed (15.5 % in Bovans and 12.5% in Saso) and age groups (13.10% in rearing, 13.51% in pullets and 15.62% in laying chickens) was relatively comparable. The difference in the isolation rate of *Salmonella* spp. among the origins where the samples were collecte, age groups, and breeds was not statistically significant (P > 0.05).

Table 5: Prevalence of *Salmonella* spp. on the basis of different associated risk parameters

Variables	**Category**	**No. of Samples Tested**	**No. of Positive samples**	**Percentage (%)**	**χ^2 (P-value)**
Site	D/Birhan	192	30	15.62	4.76 (0.18)
	Deneba	84	9	10.71	
	Keyit	62	12	19.35	
	Chacha	46	3	6.52	
Sample	C/swab	136	18	13.23	13.8 (0.003)
	Feces	130	29	22.30	
	Feed	64	4	6.25	
	Water	54	3	5.55	
Breed	Bovans	200	31	15.50	0.48(0.485)
	Saso	184	23	12.5	
Age	Rearing	145	19	13.10	0.39 (0.82)
	Pullets	111	15	13.51	
	Laying	128	20	15.62	
Flock size	50-500	104	7	6.73	10.6 (0.004)
	500-1500	194	27	13.91	
	≥1500	86	20	23.25	
Total		384	54	14.06	

4.2. Molecular Detection of *Salmonella* Typhimurium

Of the 30 isolates which showed positive growth on XLD agar plate and positive for six biochemical tests, serovar *S.* Typhimurium was molecularly confirmed in 15 isolates (50% of the PCR tested isolates). The remaining 15 *Salmonella* isolates positive for biochemical tests were negative for the amplification of *S.* Typhimurium specific gene (Spy) (Figure 4).

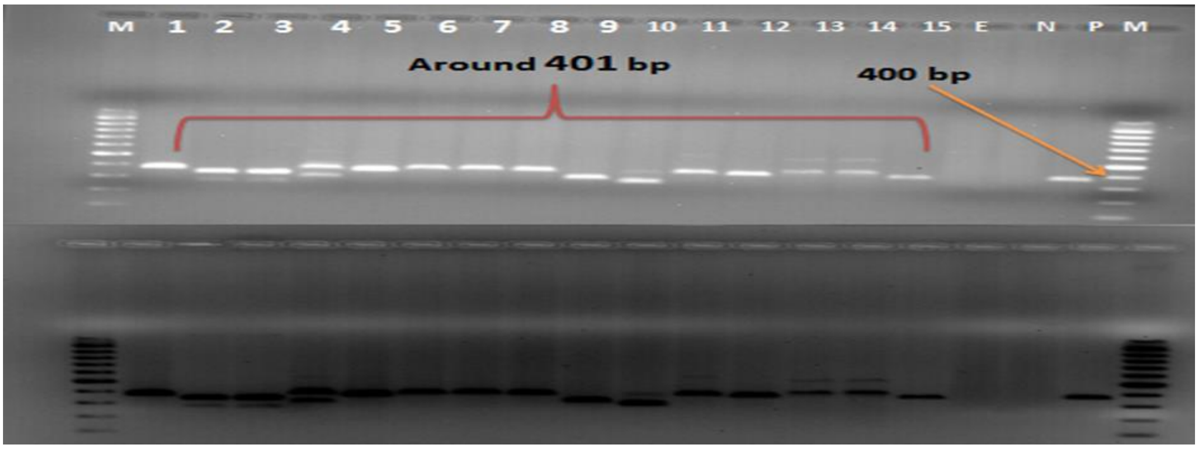

Figure 4: Electrophoresis of Spy gene for *S.* Typhimurium isolates in 1.5 % agarose gel.

M: molecular marker (100 bp); Lane 1-15: *S.* Typhimurium positive for Spy gene; Lane 16: extraction control (devoid of template DNA); Lane 17 negative control (nuclease-free water) and Lane 18: positive control (*S.* Typhimurium positive isolate).

The detection rate of *S.* Typhimurium based on sample type indicated that 44.45%, 62.50%, 33.34%, and 0% in the cloacal swab, fecal, feed, and water samples respectively. Like the bacteriological culture result of this study, the higher detection rate of *S.* Typhimurium was observed in the fecal sample followed by cloacal swab and feed samples, while none of the *S.* Typhimurium serovars were recorded from water samples. According to the present study, *S.* Typhimurium infection was recorded in 10 (76.92%), 3(30.00%), and 2(28.57%) in poultry farms which have flock sizes ≥1500, 500-1500, and ≤ 500 birds per farm, respectively (Table 6). Generally, the current study showed that the detection rate of *S.* Typhimurium was higher in laying chickens, flock size greater than 1500, and Bovans Browns' breed. The molecular detection rate of *S.* Typhimurium in this study also revealed that statistically significant association ($P < 0.05$) was observed in age groups and flock size. However, there was no statistically significant difference in detection rate of *S.* Typhimurium among the sites where the samples were collected, breeds, and the sample type ($P > 0.05$) (Table 6).

Table 6: The distribution of *S.* Typhimurium based on different risk factors (n=30).

Variables	Category	No. of isolate	Positive isolates (%)	P=Value
Age	Laying	11	9 (81.8)	0.03
	Pullet	8	2 (25)	
	Rearing	11	4 (36.36)	
Sample type	C/Swab	9	4 (44.45)	0.42
	Feces	16	10 (62.50)	
	Feed	3	1 (33.34)	
	Water	2	0 (0)	
Site	D/Birhan	16	9 (56.25)	0.47
	Deneba	6	2 (33.34)	
	Keyit	6	4 (66.67)	
	Chacha	2	0 (0)	
Flock size	≥ 1500	13	10 (76.92)	0.04
	500 -1500	10	3 (30.00)	
	≤ 500	7	2 (28.57)	
Breed	Bovans	18	12 (66.67)	0.062
	Saso	12	3 (25.00)	
Total		30	15 (50)	

4.3. Antimicrobial Resistance Profile of *S.* Typhimurium

Mono drug resistance

Antimicrobial susceptibility testing was performed for 15 PCR verified *S.* Typhimurium isolates for selected antimicrobial drugs that are commonly used in animal production and human health. Table 7 showed that antimicrobial susceptibility result of *S.* Typhimurium isolates. The antimicrobial susceptibility test of the current study revealed that all *S.* Typhimurium isolates examined were resistant or intermediately resistant to two or more antimicrobials. The higher ampicillin resistance was observed in 93.3% of *S.* Typhimurium isolates, followed by oxytetracycline (86.7%) and sulfamethoxazole (46.7%). Similarly, the isolates were found to be equally resistant to tetracycline, kanamycin, and gentamycin (40% for each). However, 100%, 73.3 %, and 66.7% of the *Salmomnella* isolates were susceptible to ciprofloxacin, gentamycin, and chloramphenicol, respectively (table 7). The intermediate resistance features of the curent finding showed that more isolates were found intermediately resistant to nalidixic acid (26.7%), tetracycline, sulfamethoxazole, and gentamycin (20% each), while none of the isolates showed intermediate resistance to erythromycin (Table 7).

Table 7: Antimicrobial susceptibility test for *Salmonella* Typhimurium isolates (n =15).

Antimicrobial agent	**Drug potency (µg)**	***Salmonella* spp. isolates**		
		Susceptible (%)	**Intermediate (%)**	**Resistant (%)**
Ampicillin (AMP)	10	0(0)	1(6.7)	14 (93.3)
Chloramphenicol (CHL)	30	10 (66.7)	2 (13.3)	3(20)
Ciprofloxacin	5	15(100)	0(0)	0(0)
Erythromycin (ERT)	15	9 (60)	0 (0)	6 (40)
Gentamycin (GNT)	10	11 (73.3)	4 (20)	0 (0)
Kanamycin (KAN)	30	7 (46.7)	2 (13.3)	6 (40)
Nalidixic Acid (NAL)	30	8 (53.3)	4 (26.7)	3 (20)
Oxytetracycline (OXT)	30	0(0)	2 (13.3)	13 (86.7)
Sulfamethazole (SXT)	25	5 (33.3)	3 (20)	7 (46.7)
Tetracycline (TET)	30	6 (40)	3 (20)	6 (40)

Multidrug resistance

The multi-drug resistant features of *S*. Typhimurium isolates are shown in table 8. Of all 15 PCR positive *S*. Typhimurium isolates tested for antimicrobial susceptibility, all isolates were resistant to two or more antimicrobials. Multi-drug resistant *S*. Typhimurium isolates showed 12 different resistant patterns for 10 antimicrobial agents tested. Out of 15 multidrug resistant isolates, 5 (33.3%) isolates were exhibited resistance to five or more antimicrobials. To eight antimicrobials, resistance was observed in 2(13.3%) isolates. Antibiogram profile also indicated that 4(26.6%) and 3(20%) of *S*. Typhimurium isolates were resistance to two and three antimicrobial agents, respectively. Whereas, only 1(6.67%) isolate was resistant to five antimicrobials (Table 8).

Table 8: Multiple antimicrobial resistance patterns of *S.* Typhimurium isolates (n =15)

Number of Antimicrobials	**Resistant pattern and number of isolates**	**Number of isolates (%)**
Two	AMP, OXT (3); AMP, KAN (1)	4 (26.7)
Three	AMP, OXT, SXT (1); AMP, OXT, ERT (1); AMP, OXT, TET (1)	3 (20)
Four	AMP, OXT, CHL, SXT (1); AMP, CHL, ETR, SXT (1); AMP, OXT, TET, SXT (1)	3 (20)
Five	AMP, OXT, ERT, TET, SXT (1)	1 (6.7)
Six	AMP, OXT, TET, KAN, ETR, NA (1); AMP, KAN, OXT, TET, SXT, NA (1)	2 (13.3)
Eight	AMP, OXT, CHL, ERT, KAN, TET, SXT, NAL (2)	2 (13.3)

AMP = Ampicillin; CHL = Chloramphenicol; ERT = Erythromycin; KAN = Kanamycin; NAL = Nalidixic Acid; OXT = Oxytetracycline; STR = Streptomycin; SXT = Sulfamethoxaz ole; TET = Tetracycline

The distribution of antimicrobial resistance due to MDR *S.* Typhimurium to all antimicrobials tested based on the origin wher the samples were obtained was 40%, 55%, and 52% in Debire Birhan, Deneba and Keyit, respectively. A higher antimicrobial resistance level was observed in Deneba followed by Keyit and Debire Birhan. Age wise distribution was found in 50%, 50%, and 45.3% in the age of ≤ 8, 8-18, and ≥ 18 weeks, respectively. In both locations and age groups, however, there is no statistically significant association (Table 9).

Table 9: The distribution of resistance to all antimicrobials tested base on origin of samples and the age groups (n=15 x 10)

Factors	**Categories**	**No. of observation**	**No. of Resistance (%)**	**X^2 (p-value)**
Location	D/Birhan	90	36(40%)	6.20 (0.184)
	Deneba	20	11(55%)	
	Keyit	40	21(52.5%)	
	Chacha	0	0%	
Total		150	68 (45.3)	
Age	≤ 8 weeks	40	20 (50%)	1.81 (0.77)
	8-18 weeks	20	10 (50%)	
	≥18 weeks	90	38 (42.2%)	
Total		150	68 (45.3%)	

Figure 5 shows that all multidrug resistant isolates of *S*. Typhimurium from Deneba were resistant to ampicillin, oxytetracycline, and sulfamethoxazole, whereas 50% of the isolates were resistant to the other antimicrobials examined (chloramphenicol, tetracycline, nalidixic acid, erythromycin, gentamycin and kanamycin). Similarly, isolates collected from Debire Birhan were highly resistant to ampicillin (100%), oxytetracycline (77.8%) and erythromycin (55.5%). In Keyit, isolates were resistant to oxytetracycline (100%), while 75% of the isolates to ampicillin, sulfamethoxazole and kanamycin. However, the association between the origins of the samples and the level of antimicrobial resistance was not statistically significant ($P > 0.05$).

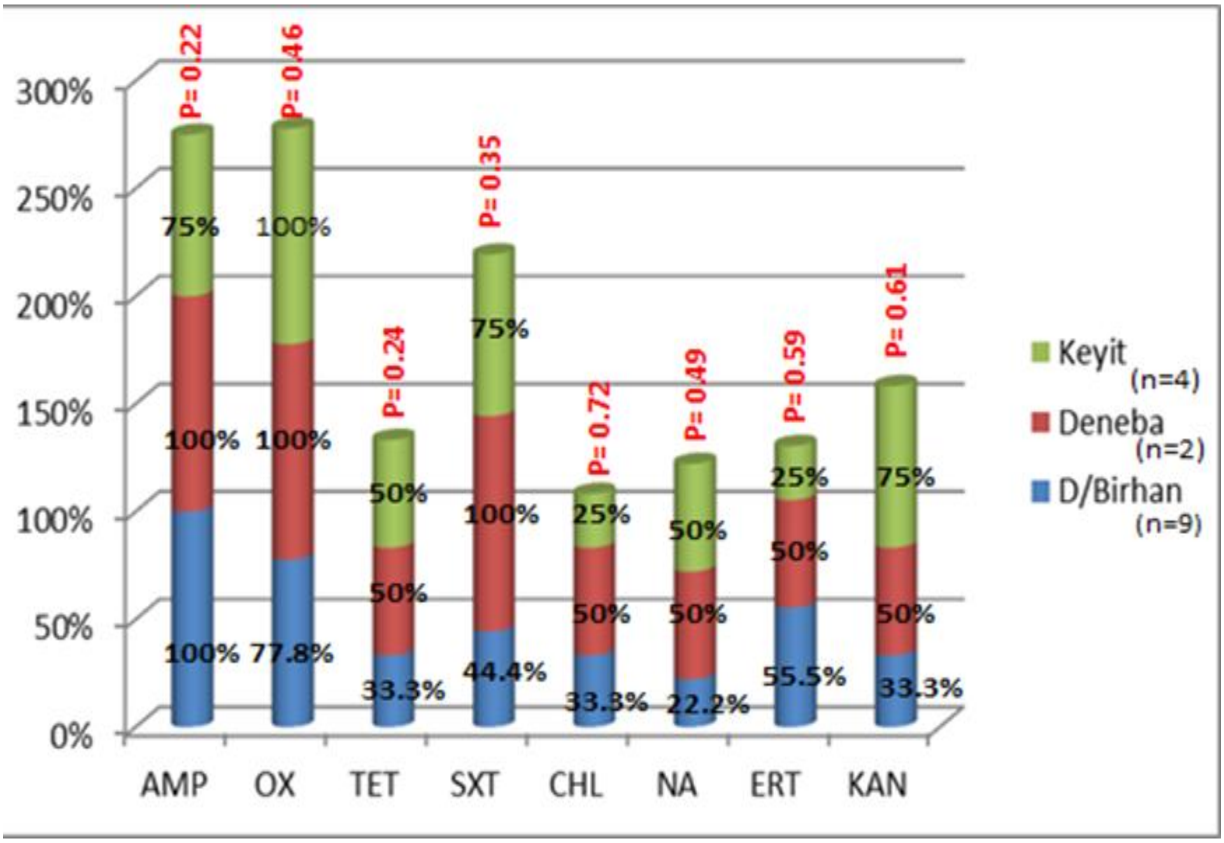

Figure 5: The resistant level of *S*. Typhimurium isolates to each antimicrobial tested based on origin where the samples were taken.

5. DISCUSSION

Isolation and Identification of *Salmonella* Isolates

Despite the importance of the poultry sector in the national economy of Ethiopia, insufficient data is a bottleneck to understanding the true prevalence, distribution, economic, and public health significance of the disease. Among the diverse bacterial and viral infections that affect poultry, salmonella infection is regarded as the most important threat to the poultry industry today. Detection, antibiogram profile and associated risk factors of *Salmonella* spp. from apparently healthy chickens and their environment were evaluated in the present study.

Based on the bacteriological culture method, 19 (73.07%) of the 26 examined poultry farms were positive for *Salmonella* spp. in agreement with the study conducted by Arkali and Çetinkaya (2020) and Mridha *et al.* (2020) who reported 73.5%, 76.7% and 68.75% isolation rate of *Salmonella* spp. at farm level, respectively. Farm level prevalence of this finding is higher than Eguale (2018), Sharma *et al.* (2021) and Odoch *et al.* (2017), reported 14.6%, 55% and 20.7%, respectively. The differences in the isolation and identification rate could be attributed to factors such as poultry management practices, housing system of chicken, and hygienic condition. The higher isolation and identification rate may be associated to poor diseases prevention and control effort in the studied poultry farms. In connection to this, all farms investigated in the present study were kept chickens in a floor based housing system. It is well recognized that living in unsanitary settings and ground housing systems can all contribute to wide spread of salmonella infection.

The overall isolation rate of *Salmonella* in the present study from biochemically confirmed isolates was 14.06%. This finding is relatively in congruence with the previous report of Abdi *et al.* (2017), Nabi (2017), and Yeh *et al.* (2018) who found 16.7%, 16% and 14.2% overall prevalence of *Salmonella* spp., respectively. However, this finding was lower than the 42.7%, 21.3% and 28.6% isolation rates reported from Jimma (Kindu and Addis, 2013), Ugandan (Odoch *et al.*, 2017) and Egypt (Elkenany *et al.*, 2019), respectively. It was also found higher than studies conducted in Hawassa (8%), Haramaya (2.7%), Ambo and Holeta town (5.7%), Nigeria (2%), and India (7.4% and 6.1%) reported by Aragaw *et al.* (2010), Kemal *et al.* (2016), Gebremedhin *et al.* (2021), Chinasa *et al.* (2019), Waghamare *et al.* (2018) and Samanta *et al.* (2014), respectively.

The difference in overall sample level isolation rate between prior research and the current study can be explained partly variation in nature of samples utilized, study area, flock size, and poultry farm concentration. Larger number of medium sized farms, the collection of environmental samples from the cooler area during the dry season of the year, and a lower concentration of poultry farms in the area, all of which may contributed to a lower isolation rate (Hailu and Kebede, 2016). Higher isolation rate of *Salmonella* spp. has been observed in the area of the warmer region and in areas where highly concentrated with larger numbers of poultry farms (Eguale, 2018). The higher isolation and identification rate observed of this finding probably due to a deprived management system, poor biosecurity measures practice, lack of regular vaccination (Revolledo and Ferreira, 2012a).

In the current study, location, age, breed, flock size and sample type have been assessed as triggering factors for the isolation and identification rate of *S.* Typhimurium. Despite the higher rate of isolation and identification using standard bacteriological culture method was recorded in Keyit (location), laying chickens (age), and Bovans brown (breed), there was no statistically significant association with the rate of isolation and identification of *Salmonella* spp. However, the isolation rate of *Salmonella* found in the current study was significantly varied depending on the type of samples and flock size.

Regarding the sample type, significantly higher and lower isolation rate was recorded from the fecal sample (22.30%) and water sample (5.55%), respectively. This finding is in line with the study conducted elsewhere in Ethiopia by Abunna *et al.* (2018), Abdi *et al.* (2017), Dagnew *et al.* (2020) and Ketema *et al.* (2018) who reported relatively larger percentage of *Salmonella* spp. were isolated from fecal samples than other sample settings. Comparable to this, research from Nigeria showed that the higher *Salmonella* isolation and identification rate was recorded from the fecal sample (23%) and having a relatively lower isolation rate in water sample (15.1%) (Fagbamila *et al*., 2017). However, the current finding was contrasting with Sharma *et al*. (2021) who reported higher *Salmonella* isolates from the water sample (27.5%) than the fecal sample (10.6%). The higher isolation and identification rate of *Salmonella* spp. in fecal droppings might be due to the fact that gastrointestinal system thought to be a potential source of contamination during intermittent shedding of pathogen with feces from carrier chickens (Jajere, 2019). On the other hand, the reduced recovery rate of *Salmonella* isolates in feed and water samples compared to fecal droppings and cloacal swabs suggest that feed and water used in chicken farms might be safe or due to small sample size. Overall, feces, particularly pooled feces, had greater *Salmonella* isolation rate than cloacal swabs and other environmental samples (Kotton *et al.*, 2006).

The isolation and identification rate of *Salmonella* spp. from bacteriological culture of the current study was significantly higher (23.25%) in poultry farms with large flock size ($\geq$1500 birds/farm) which is granted with the previous finding elsewhere in Ethiopia and in the world (Eguale, 2016; Odoch *et al*., 2017; Eguale, 2018). A significant difference in *Salmonella* occurrence among farms of different flock sizes was also observed in research conducted in Adama and Mojo poultry farms (Dagnew *et al.*, 2020). The higher isolation rate in large sized farms is in concordance with the fact that farm size has been related with feeding and other managemental activities. large sized poultry farms tend to attract many visitors, use multiple sources to obtain poultry feeds, chickens and other supplies which make difficult to keep adequate hygienic standards (Barua *et al.,* 2012). Furthermore, large sized poultry farms require many workers to perform routine activitiesroutin activities like feeding, watering, cleaning and vaccinations, some of them coming from outside the farms and increase the chance of *Salmonella* contamination. In addition to favoring stress to chickens, which increases continuous shedding of *Salmonella* from carriers, intensification increase animal to animal contact and allows easy dissemination of the *Salmonella* spp. within the farm (Eguale, 2018; Jajere, 2019).

Molecular Detection of *Salmonella Typhimurium*

Salmonella Typhimurium is one of the most threatening serotypes for public health because of its association with antibiotic resistance (Olobatoke, 2015). The detection rate of *S.* Typhimurium 50% was found slightly comparable to 43.35% and 40% detection rate of *S.* Typhimurium reported by Abdellah *et al.* (2009) and Adhikari *et al.* (2018), respectively. This finding is very much higher than Merera (2018) (18.2%), Jamshidi (2009) (1.6%), Arkali and Çetinkaya (2020) (9.4%), El-Aziz (2013) (33%), and Pritha *et al.* (2020) (15.38%). However, the higher result had been reported by other authors (Olobatoke, 2015; Nyabundi *et. al.,* 2017). The nature of the serovar tested coupled with other managemental and environmental related factors could contribute for the disparity. Serovar typhimurium is well known for its capacity to infect a wide range of animals and for its ability to survive in the environment for long periods, making it one of the most common causes of salmonellosis (Rabsch *et al.,* 2002). The distribution of *Salmonella* serovars also varies geographically and change in time (Waghamare *et al*., 2018).

Molecular detection rate of *S*. Typhimurium isolates revealed that chickens which had ≥18 weeks of age (laying chickens, 81.8%) had a significantly (*P* = 0.05) higher detection rate followed by rearing (less than 8 weeks of age, 36.36%) and pullets (8-18 weeks of age, 25%). This finding is in good harmony with the study conducted by Kindu and Addis (2013) in Jimma Town. However, this finding disagrees with the work done by Abdi *et al.* (2017). He concluded that young age groups had a significantly higher prevalence followed by layers. The discrepancy in these studies could attributable to the difference in the age and the size of the flock in the farms studied and other management factors. The increased isolation rate in laying chickens and rearing chickens may due to the fact that chickens remain a carrier of *S*. Typhimurium with persistent shedding to the external environment once infected at a young age, while younger chickens are highly susceptible and unable to clear infection (Mkangara *et al.*, 2020). Furthermore, physiological stress resulted from egg production might reduce immune response in laying chickens (Landers *et al.,* 2005). Although the mechanism was not understood, age is associated with increased morbidity and mortality from *S*. Typhimurium infection (Ren *et al*., 2009).

Unlike flock size, there was no statistically significant variation in the detection rate of *S*. Typhimurium based on sample type using conventional PCR. Similarly, age group was not significantly associated with the isolation and identification rate of *Salmonella* spp. which is true in molecular detection of *S.* Typhimurium. The sample size difference between the two methods may contribute to explain the discrepancy, as the bacteriological culture method used larger sample size (384 samples) than conventional PCR (30 isolates). On top of this, conventional PCR is highly sensitive and specific diagnostic method than bacteriological culture methods (Gwida, and Al-ashmawy, 2014; Jinu *et al*., 2014,).

Antimicrobial Resistance of *Salmonella. Typhimurium*

The emergence and spread of antimicrobial resistant *Salmonella* strains is a serious concern in the fields of public health, animal health, and food safety. The finding of antimicrobial susceptibility investigation in the current study indicated that all *S*. Typhimurium isolates (n=15) were resistant or intermediate resistant to at least two tested antimicrobials. This finding is closely matched to a study conducted in Ethiopia (Eguale *et al.,* 2016; Geresu *et al.,* 2021) where all *Salmonella* isolates were resistant to at least one antimicrobials tested. However, this finding is higher than Taddese *et al*. (2019) and Odoch *et al. (*2017) who reported 58% and 72.72% of the isolates were resistant to at least one antimicrobial agent tested, respectively. The higher resistance pointed out in the present study could have resulted from irrational use of antimicrobials tested in poultry farms since there is a lack of advocacy and monitoring of antimicrobial utilization (Gebremedhin *et al.*, 2021b). Indiscriminate use of antimicrobials without prescription and improper dispensing might favor selection pressure that increase the advantage of maintaining resistance genes in bacteria (Ejo *et al.*, 2016). The other possible reason behind this discrepancy could be the number of serotypes investigated. In this regard, only *S.* Typhimurium was tested for certain drugs in the current study. Odoch *et al.,* (2017) and Taddese *et al.* (2019) on the other hand, investigated serotypes other than *S.* Typhimurium.

The antibiogram profile of the current research indicated that isolates of *S.* Typhimurium showed resistance to ampicillin, oxytetracycline, sulfamethoxazole, tetracycline, and erythromycin with resistance rates of 93.3%, 86.7%, 46.7%, 40%, and 40%, respectively. The isolates were relatively higher resistant to ampicillin (93.3%) and oxytetracycline (86.7%). This finding was in agreement with the previous studies conducted by Abdi *et al.* (2017), Garedew *et al.* (2015), Geresu *et al.* (2021) and Chibuzo and Okike (2021) who reported that 97.8%, 100%, 100% and 90% of *Salmonella* isolates were resistant to ampicillin, respectively. The emergence of *Salmonella* spp. resistance to above-mentioned antimicrobials could be due to indiscriminate and/or long-term use of these two drugs in both the livestock and human health sectors, as a result of uncontrolled accessibility and affordability in Ethiopian drug vendors (Abunna *et al.*, 2016; Gebremedhin *et al.*, 2021b).

The widely use of feed additives with sub-therapeutic doses as a growth promotant (Adhikari *et al.*, 2018) and prophylaxis treatment (Chinasa *et al.*, 2019) could also be the most likely reason. The higher ampicillin resistance also supported by another investigator (Arkali and Çetinkaya, 2020) who found that 67% (4/6) *S.* Typhimurium isolates showed a gene responsible for ampicillin resistance. Contrary to these, Dagnew *et al.* (2020) was reported lower resistance to ampicillin from Adama and Mojo poultry farms. For this contradiction, the author suggests that this could be resulted from the less use of ampicillin lead to new strains of *Salmonella* serovars circulating in those areas with loss of genetic markers.

Due to its broad range of antibiotic properties, oxytetracycline is the popular and widely used antibiotic and associated with resistance in veterinary sectors globally (Hiko *et al.*, 2018). In Ethiopia, oxytetracycline has been one of the most widely used drugs for the treatment of infectious diseases including Salmonellosis, and as prophylaxis from day-old chicks to laying hens and which contributed to a selection of Salmonella strains resistant to oxytetracycline (Dagnew *et al.*, 2020). Antimicrobialsis widely used in Ethiopian poultry farms, as practically all of them utilize antimicrobials such as oxytetracycline and sulfonamides for therapeutic or preventative purposes when one or more sick birds are present in the flock (Eguale, 2018). Unlike oxytetracycline, resistant development in ampicillin (93.3%), chloramphenicol (20%), and nalidixic acid (20%), which often used in humans suggest that antimicrobial resistance genes are transmitted across species. In general, the high resistance to oxytetracycline and sulfamethoxazole shown in the present research given that these antimicrobial drugs are frequently utilized in most farms for the treatment of most bacterial infections.

On the other hand, this finding revealed that none of the isolates resistant to ciprofloxacin, which is consistent with the finding of Merera (2018), Taddese *et al.* (2019), Chibuzo and Okike (2021), and Abdi *et al.* (2017) who reported none of *Salmonella* isolates resistant against ciprofloxacin. In contrast to these, studies conducted by Odoch *et al.* (2017) and Siddiky *et al.* (2021) revealed that 50.0% and 100% of the isolates were resistant to ciprofloxacin, respectively. Similarly, 31.1% and 54.5% of isolates were found to be resistant to ciprofloxacin and gentamycin, respectively (Biffi *et al.*, 2014; Abdi *et al.*, 2017). The absence or limited use of ciprofloxacin and gentamycin is well known to results in the lower resistance rate of *S*. Typhimurium. According to a survey of chicken farm owners, the use of ciprofloxacin and other quinolone antimicrobials was not a common practice in Ethiopian veterinary treatment (Eguale *et al.*, 2016).

The multidrug resistant features of the present study is alike to the finding of Geresu *et al.* (2021), Abdi *et al.* (2017) and moderately comparable with Eguale *et al.* (2016). The more recently 80.91% and 78.1% multidrug resistance findings in Bangladesh (Mridha *et al.*, 2020) and Siddiky *et al.*, (2021), respectively were also comparable. According to MDR features 4(26.7%) isolates were resistant to two antimicrobials tested, three of which had the same resistance pattern. Closely comparable finding (23.38%) to this resistance feature was reported by Pritha *et al.*(2020). The higher prevalence of multidrug-resistant *S*. Typhimurium recovered may be due to the factors explained before. Furthermore, *S*. Typhimurium can naturally resist a variety of antimicrobial drugs and develop multi-drug resistance against many antimicrobial agents (Yang *et al.*, 2020).

6. CONCLUSIONS AND RECOMMENDATIONS

The finding of the current study explicitly provide information and data on the isolation and identification rate of *Salmonella* species, detection rate and antibiogram profile of *S*. Typhimurium and assessment of the associated risk factors of *Salmonella* from poultry farms in and around Debire Birhan. The higher isolation and identification rate of *Salmonella* species was identified in both farm and sample level *Salmonella* investigation. The higher detection rate of *S*. Typhimurium encountered in the current study suggests that this serovar is the highly circulating serovars in the areas. Flock size, sample type, and age group were among the risk factors that were significantly associated with the detection rate of *Salmonella* spp. In work done to know the antimicrobial susceptibility profile of certain antibiotics, the higher prevalence of multidrug resistant *S*. Typhimurium were determined. Therefore, the higher isolation and identification rate of *Salmonella* isolates and the appearance of multidrug resistant *S*. Typhimurium as disclosed by the current findings instigates to serious economic, veterinary and public health problems.

Therefore, in line with the above concluding remarks, the following recommendations are forwarded.

- Further studies ought to be conducted on the detection of multidrug resistant strains of *Salmonella* other than *S*. Typhimurium and their resistant gene. In connection to this, comprehensive molecular characterization or serotyping of *Salmonella* isolates should be necessary to ascertain circulating *Salmonella* serovars in the study area.
- Implementing *Salmonella* prevention and control strategies, exercising rational drug use policy along with monitoring illegal antimicrobial use at various point of livestock health and production sectors should be needed.
- Regular surveillance and monitoring of *Salmonella* infections and its antimicrobial resistance patterns, riase awareness among poultry producers, poultry farm attendants and the community for better farming practices and antimicrobial resistance threats should be carrid out.
- Attention should be given while selection of effective antimicrobials during treatment of *Salmonella* infection at various point of livestock productions for proper handling of multiple drug resistant *Salmonella* strains.

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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 121^0C 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.

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 121^0C 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 +4^{0}C 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.

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

Composition	**g/L**
pancreatic digestion of casein	17
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 at121^{0}c 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: Media and 0.5 McFarland standards preparation for drug susceptibility testing

a: Mueller-Hinton Agar (CM 0337, OXOID, Basingstoke, England)

Composition	**g/l**
beef, dehydrated infusion	300.00
casein hydrolysate	17.5
Starch	1.5
Agar	17.00

Preparation:

Suspend 38 grams in 1000 ml of distilled water. Bring to boil to dissolve the medium completely. Sterilize by autoclaving at 121℃for 15 minutes. PH: 7.3 + 0.1 at 25℃

b. 0.5 McFarland standards

Composition	**Amount**
1.17% BaCl.2H2O solution	0.05 ml
0.36N of 1% sulfuric acid (H2SO4).	9.95ml

Preparation:

Add approximately 85Ml of 1% H2SO4 to a 100ml of a volumetric flask, using a 0.5ml pipette add 0.05ml of 1.17% BaCl.2H2O dropwise to the 9.95ml of 1% H2SO4 while constantly swirling the flask. Bring to 100ml with 1% H2SO4. Place a magnetic stirring in the flask and place on the magnetic stirrer for approximately three to five minutes. Examine solution visually to make certain it appears homogeneous and free of visible clumps.

Annex 3: 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 gm

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 casting 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.

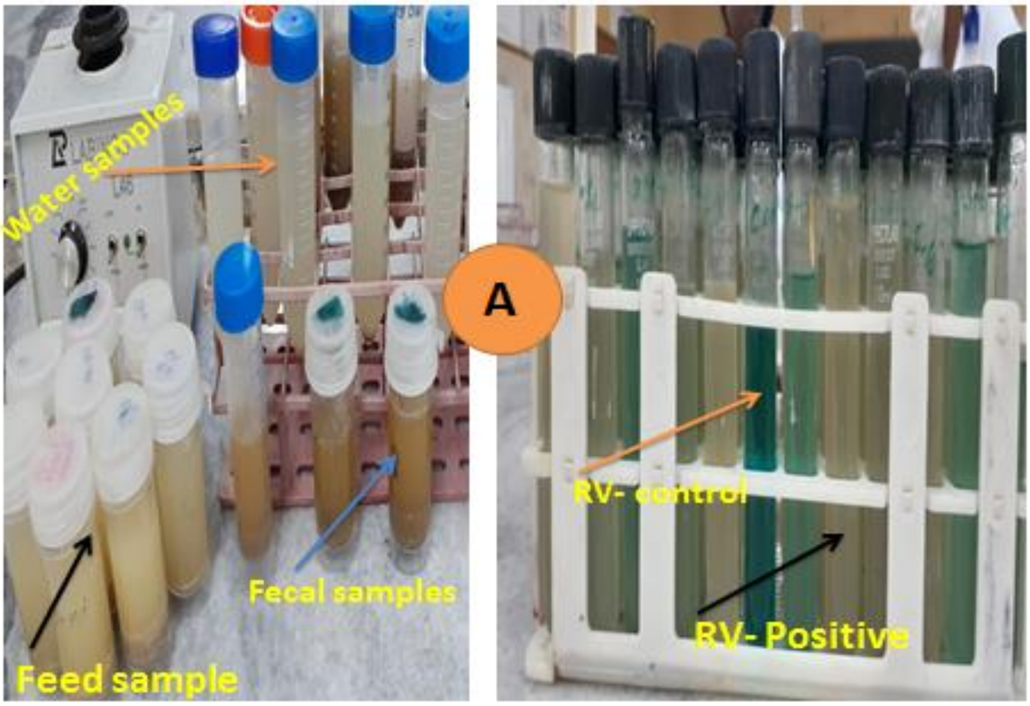
c. Molecular marker

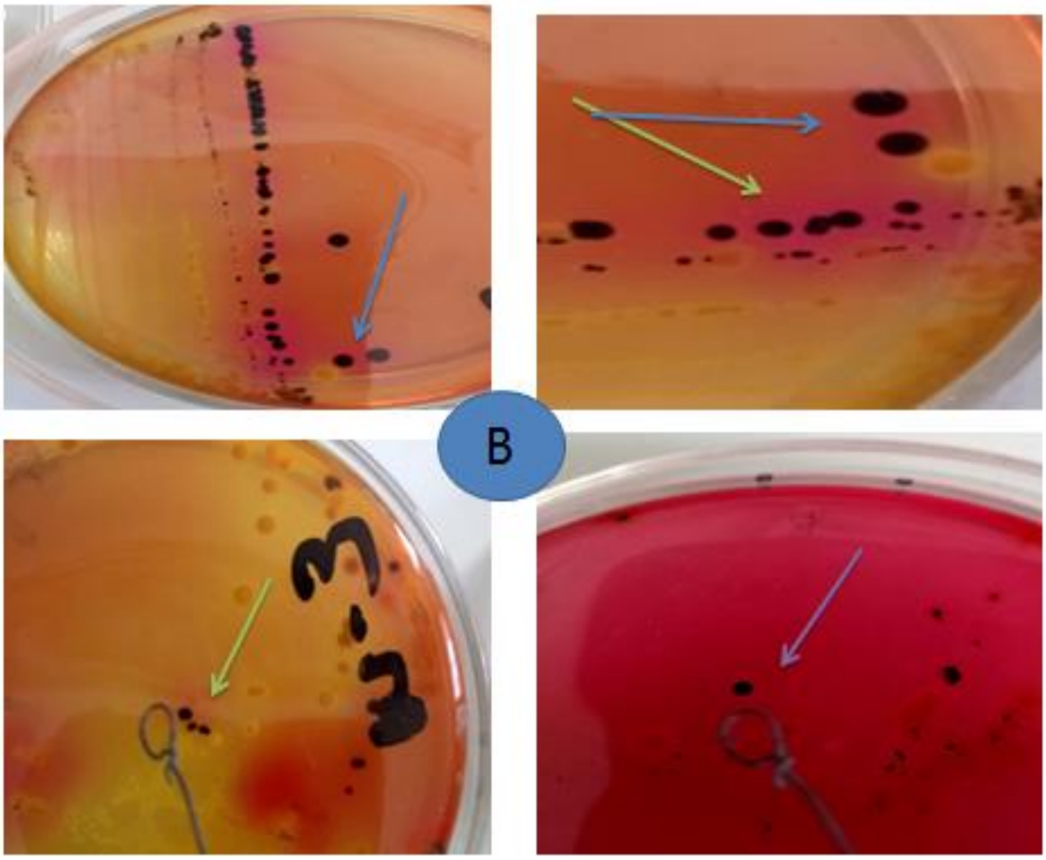
Composition	**Amount**
loading buffer	475 µl
Gel Red	25 µl
PCR water	265 µl

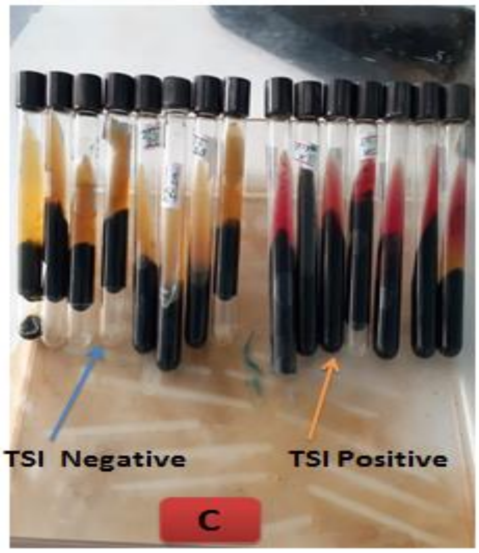
Procedure:

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.

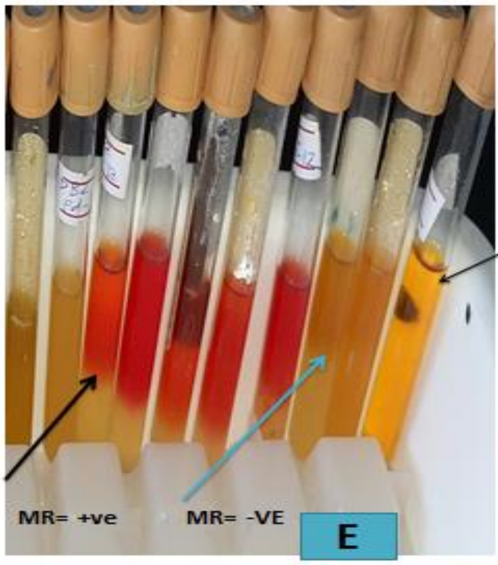
Annex 4: *Salmonella* colonies, biochemical results and antimicrobial inhibition zone

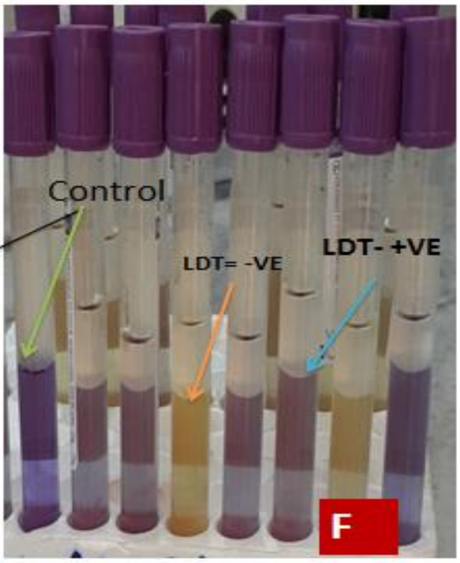


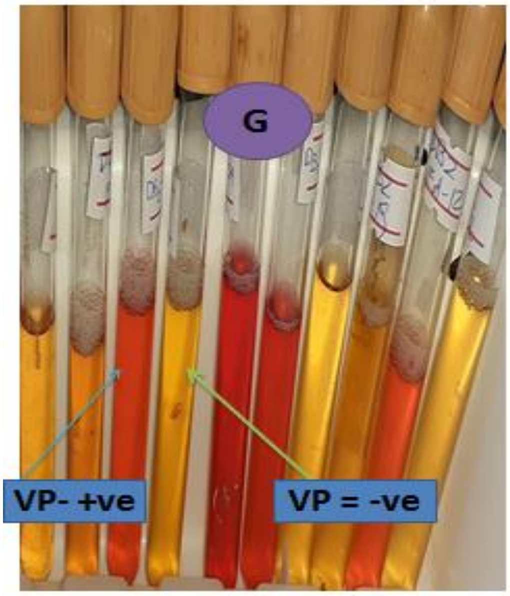


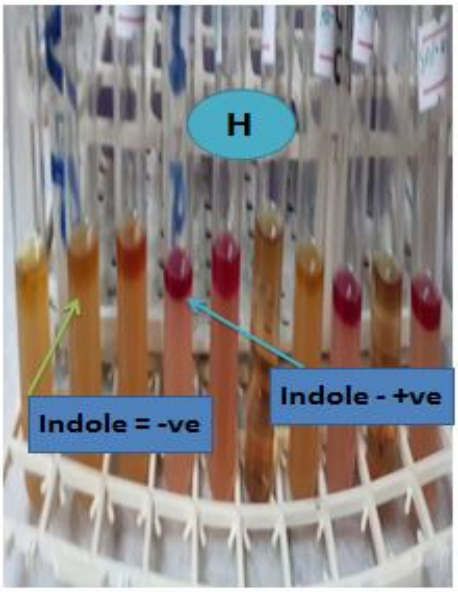

TSI Negative
TSI Positive
C

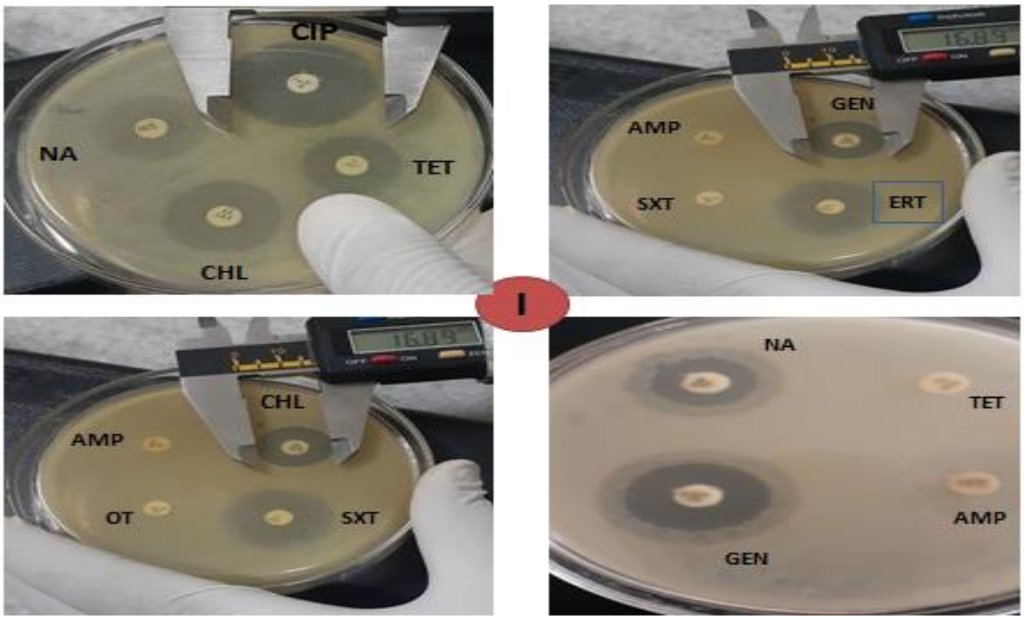

Simon's citrate Test
D

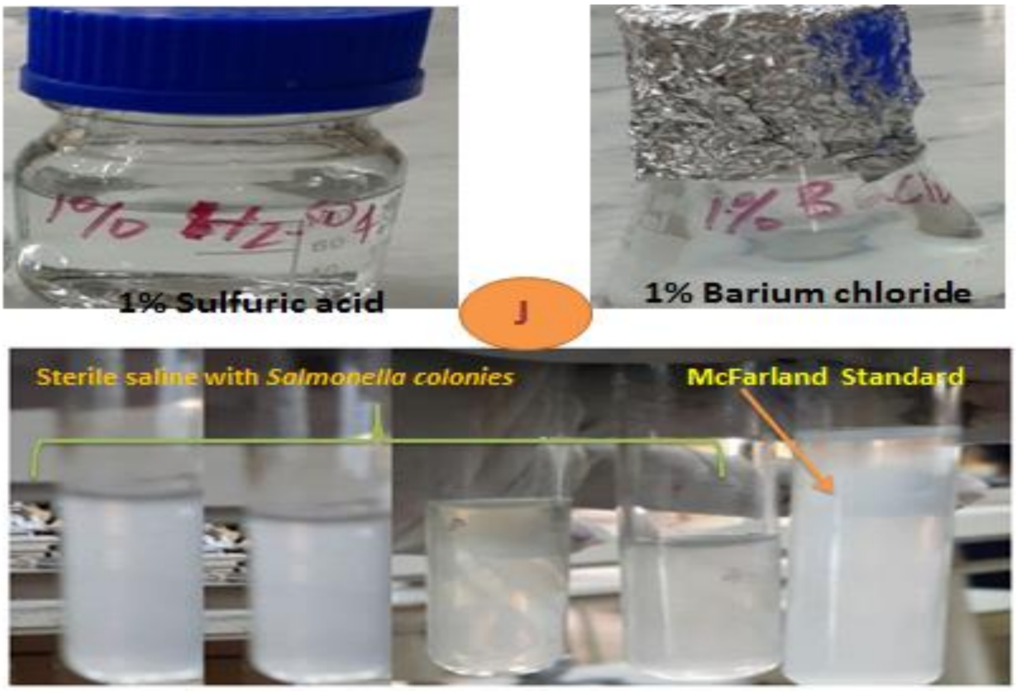

MR= +ve
MR= -VE
E


Control
LDT= -VE
LDT- +VE
F


G
VP- +ve
VP = -ve


H
Indole = -ve
Indole - +ve





Annex 5: Data collection and laboratory result recording Sheets

a. Data collection sheet

ID No	Site	Farm name	Age	Breed	Farm size	Sample type
1						
2						
3						

b. Bacteriological tests result recording sheet

ID No.	RV	Plating media		Biochemical test results					PCR
		XLD	BGA	TSI	SCT	Indole	MR-VP	LDT	
1									
2									

XLD = Xylose Lysine Decarboxylate Test; BGA = Briliant Green Agar; TSI = Triple Sugar Iron Agar; SCT = Simon's Citrate Agar; MR-VP = Methyl Red and Vagos Prosker; LDT = Lysine Decarboxylation Test; PCR = Polymerase Chian Reaction

c. Drug sensitivity result recording sheet of Salmonella isolates in µg

Isolates	AMP (10)	CHL (30)	CIP (5)	GNA (10)	KNA (30)	NA (30)	OXT (30)	SXT (25)	TET (30)	ERT (15)
1										
2										

Annex 6: Antimicrobial Susceptibility Breakpoints for *Enterobacteriaceae.*

Types of antimicrobials	Concentration (µg/disc)	Susceptible	Intermediate	Resistant
Ampicillin (AMP)	10	≥17	14 – 16	≤13
Chloramphenicol (CHL)	30	.≥ 18	13-17	≤12
Erythromycin (ERT)	15	≥ 23	14-22	≤ 13
Gentamycin (GNT)	10	≥15	13 – 14	≤12
Kanamycin (KNA)	30	≥18	14 -17	≤13
Nalidixic Acid (NA)	30	≥19	14 – 18	≤13
Oxytetracycline (OX)	30	≥16	12 – 16	≤12
Ciprofloxacin	5	≥31	21-30	≤ 20
Sulfamethazole (SXT)	25	≥16	11 – 15	≤ 10
Tetracycline (TET)	30	≥15	12 – 14	≤11

Source*:* Clinical and laboratory standard Institute (CLSI, 2016)

Annex 7: Different Laboratory Activities during the Study Period