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ISOLATION, MOLECULAR DETECTION, AND ANTIMICROBIAL SENSITIVITY TEST OF *SALMONELLA* ISOLATES IN BROILER CHICKEN PRODUCTION SYSTEM IN AND AROUND ADAMA TOWN, CENTRAL ETHIOPIA

MSc Thesis

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MSc Program in Veterinary Microbiology

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BISHOFTU, ETHIOPIA.

ISOLATION, MOLECULAR DETECTION AND ANTIMICROBIAL SENSITIVITY TEST OF *SALMONELLA* ISOLATES IN BROILER CHICKEN PRODUCTION SYSTEM IN AND AROUND ADAMA TOWN, CENTRAL ETHIOPIA

MSc Research Thesis Submitted to the College of Veterinary Medicine, Addis Ababa University in Partial Fulfillment of The Requirements for The Degree of Master of Veterinary Science in Veterinary Microbiology

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STATEMENT OF THE AUTHOR

First, I declare that this thesis is my authentic work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture, and is deposited at the University/College library to be made available to borrowers under the rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
CDC	Center for Disease Control and Prevention
CSA	Central Statistics Agency
DNA	Deoxyribose Nucleic Acid
FAO	Food and Agriculture Organization
FT	Fowl Typhoid
HACCP	Hazard Analysis Critical Control Point
MAPK	Mitogen-Activated Protein Kinase
MDR	Multi-Drug Resistance
NCCLSI	National Committee for Clinical Laboratory Standards
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PD	Pullorum Disease
PCR	Polymerase Chain Reaction
RVS	Rappaport-Vassiliadis Soy peptone broth
SCV	Salmonella-Containing Vacuole
SPI	Salmonella Pathogenicity Island
SPV	Salmonella Plasmid Virulence
TAE	Tris-acetate-EDTA
TLR	Toll-Like Receptor
TSI	Triple sugar Iron agar slant
T3SS	Type 3 Secretion Systems
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate agar

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ABSTRACTS

Ethiopia is one of the leading countries in having a large chicken population in Africa. However, different constraints are affecting the productivity of the chickens in the country. Salmonellosis is one of the prioritized bacterial diseases affecting poultry in the country and poultry meat is the major source of non-typhoidal salmonellosis in humans. As the result, a cross-sectional study was conducted from November 2020 to July 2021 in purposively selected broiler chicken farms found in and around Adama town with the aim of isolation, molecular detection, and antimicrobial susceptibility profiling of *Salmonella* from broiler chickens. A total of 380 samples (cloacal swab, n=260; pooled fresh fecal dropping, n=30; pooled litter sample, n= 30; pooled feed sample, n=30; and pooled water swab, n=30) were randomly collected from 6 purposively selected broiler chicken farms. Standard bacteriological techniques were employed to conduct isolation and biochemical test confirmation of *Salmonella* followed by molecular confirmation using conventional PCR and the drug sensitivity testing was done for 10 antimicrobials by using disk diffusion methods according to CLSI guidelines. Out of the total 380 samples collected, 88 (23.2%) were positive for *Salmonella*. Based on the potential effects of sample type, the likely hood of isolation of *Salmonella* was 6.91 times significantly higher in the fecal sample as compared to the other types of samples. A total of 42 representative samples out of 88 (~50%) biochemically positive *Salmonella* were subjected to molecular detection and 10 (23.8%) of the isolates were positive to *S.* Enteritidis using the *Sdf*I gene. From the PCR positive *S.* Enteritidis profiled for antimicrobial susceptibility, the highest resistance (90%) was observed in Ampicillin followed by Tetracycline (80%). Multi antimicrobial resistance (MAR) was recorded in 8(80%) of the isolates; indicating the irrational use of antibiotics in broiler chickens which leads to the potential source for MAR *Salmonella* infection in both animals and humans. Therefore, improvement of knowledge of farmers and judicious use of antimicrobial and restriction of getting drugs without a prescription is crucial and mandatory. Further, molecular confirmation and sequencing should be done to identify the gene that plays a role in resistance.

Key Words: *Adama, Antimicrobial sensitivity, Broiler chicken farm, Multi-antimicrobial Resistance, Prevalence, S. Enteritidis*

1. INTRODUCTION

Ethiopia is one of the leading countries in having a large chicken population in Africa with an estimation of 59.5 million. The chickens breed in the country comprising 91% local breed and the remaining 9% are hybrid and exotic breeds. The rearing of chicken accounts for the country's economy by improving food and nutrition security to the most vulnerable sections of the urban resource-challenged. As well as the source of income for many Ethiopians whose livelihood depends on it (Alemneh and Getabalew, 2019; Asfaw *et al*., 2019).

Poultry diseases are the main problem for compromised productivity and reduction of total chicken numbers (Alemneh and Getabalew, 2019). For instance, as of CSA report, about 32 million birds died due to disease, and close to 30 million birds were lost due to causes other than a disease, mainly predators in 2016 (FAO, 2019).

From the common diseases found in poultry farm, salmonellosis is one of a bacterial disease. It is an enteric disease of different host species that live in the intestinal tracts of warm-blooded and cold-blooded animals (Todar, 2008). Salmonellosis has zoonotic importance, which is responsible for food-borne infection of humans worldwide (Wibisono *et al*., 2020). It also has economic significance associated with high mortality and reduced productivity of chickens (Uro, 2019).

Salmonellosis is caused by the genus *Salmonella*, which is a short bacillus (rod-shaped), Gram-negative, facultative anaerobes, and non-spore forming bacteria. They are also motile that possess peritrichous flagella except for serovar *Salmonella* Pullorum and *Salmonella* Gallinarum (Quinn *et al*., 1999). The genus *Salmonella* embraces two species, *Salmonella enterica, and Salmonella bongori.* The type species *Salmonella enterica* has six subspecies, that also further classified into more than 2600 serovars (Gal-Mor *et al*., 2014).

From various *Salmonella* serotypes associated with poultry infection, the avian host-specific *Salmonella* has the ability to colonizing, infecting live birds, and transmitted between birds by both vertical and horizontal transmission (Foley *et al.*, 2008). *Salmonella* Gallinarum and *Salmonella* Pullorum are among the host-specific serovars to result in fowl typhoid and

pullorum disease respectively in poultry farms and leads to considerable economic losses in many developing countries (Foley *et al.*, 2008).

Birds also infected with non-host specific *Salmonella* strains including *S.* Typhimurium and *S*. Enteritidis without showing the sign of infection. But become a common source of human *Salmonella* infections through consumption of their product especially eggs (Geetha and Palanivel, 2018). This contributes to 78 million foodborne illnesses and 230,000 deaths per year, worldwide (Bonifait *et al*., 2021).

Salmonella infections are medically treated by using an antimicrobial agent such as ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole. Improper use of antibiotics in both sectors (human health and agricultural sectors) results in resistant pathogens (Meneses, 2010). The emergence of antimicrobial resistance in *Salmonella* was first reported in the early 1960s for single antibiotic chloramphenicol (Saha *et al*., 2020).

Currently, most of the *Salmonella* strains have become multi-antimicrobial resistant in which they are resistant to multiple antibiotics. These antibiotics were being used in the promotion of food animal growth and in veterinary medicine to treat bacterial infections in animals. This results in a high risk of multi-antimicrobial resistant (MAR) *Salmonella* strains. There is a likely hood of these strains to be transmitted from animals to humans, through consuming contaminated food of animal origin (Threlfall, 2002). MAR *Salmonella* strains became a serious health problem worldwide and as a tradition, the habit of eating raw or undercooked animal products is one of the attributes that aggravates the transmission of the MAR *Salmonella* strain in Ethiopia (Gutema *et al*, 2021).

Considering the high exposure in the country, several studies have been conducted in different parts of Ethiopia to determine the occurrence of *Salmonella* and the proportion of MAR *Salmonella* isolates in poultry farms, which implies a significant public health risk of poultry-associated Salmonellosis (Bekele, and Ashenafi, 2010; Aragaw *et al*, 2010; Abdi *et al*., 2017; Eguale, 2018; Asfaw *et al*, 2020;). Even though there are many studies conducted in various parts of the country, there is little information on the presence of *Salmonella* in broilers chicken farms, explicitly utilizing molecular tools to detection of the circulating

Salmonella serotypes, and their antimicrobial susceptibility profiles among broiler chickens and chicken farms.

Therefore, the objectives of the present study were:

- Isolation of *Salmonella* from cloaca swab, feces, litter, feed, and water samples from the selected broiler chicken farms in and around Adama town, central Ethiopia,

- Molecular confirmation of the isolated *Salmonella* serotype from the selected poultry farms in and around Adama town, Ethiopia and

- Determination of the antimicrobial susceptibility profiles for the isolated and molecularly confirmed *Salmonella* serotypes.

2. LITERATURE REVIEW

2.1. Etiology

Salmonella is Gram-negative bacilli, that are facultative anaerobes, intracellular, nonsporulating, and straight-rod shaped bacteria (Todar, 2008). The bacterial cytoskeleton composed of an actin-like protein maintains the structure of the rod. They are 2–5 mm long and 0.8–1.5 mm wide on average. Members of the genus *Salmonella*, excluding SG and SP, are motile through peritrichous flagella. They belong to the same family as *Escherichia coli* in the Enterobacteriaceae family**.** They are chemoorganotrophic which metabolize nutrients through both respiratory and fermentative pathways (Jajere, 2019).

2.2. General Characteristics of Genus *Salmonella*

They could grow at temperatures varying from 8 to 45 °C (optimum temperature 37 °C), at pH levels ranging from 4.0 to 9.5 (optimum pH 6.5–7.0), and at water activity levels as low as 0.94. Some strains can grow at 54°C, while others can grow at 2–4°C (Percival and Williams, 2014).

Salmonella is positive to catalase and methyl-red tests, negative to oxidase or does not produce the enzyme cytochrome oxidase, indole production, and Voges-Proskauer tests. The bacteria ferment glucose, mannitol, and sorbitol to generate acids and gas. They do not ferment Lactose sugar except *S.* Arizonae which can produce bisulfates by fermenting lactose. They may also ferment sucrose, but not adonitol, and do not hydrolyze urea or deaminate phenylalanine, and utilize citrate as a sole source of carbon. They produce lysine and ornithine decarboxylases except for *S.* Paratyphi A and *S.* Typhi (Percival and Williams, 2014).

A large majority, except for a few serovars, like *S.* Paratyphi A and *S*. Choleraesuis, produce hydrogen sulfide. Based on this valuable specific property a variety of selective and differential media for culture, separation, and presumptive recognition of *Salmonella* has been invented (Jajere, 2019). *Salmonella* is found in both warm-blooded and cold-blooded

animals' intestinal tracts. Some species are ubiquitous, while the other species are specifically adapted to a particular host (Todar, 2008).

2.3. Taxonomy and Nomenclature

Salmonella was discovered in 1855 for the first time by Theobald Smith, from the intestine of pigs that infected with classical swine fever. Based on sequence analyses difference of 16S rRNA the genus *Salmonella* are classified into two species, *Salmonella enterica* and *Salmonella bongori* (Eng *et al*., 2015).

The type species *S. enterica* is comprising six subspecies, that indicated by Roman numbers. Thus are *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and *S. enterica* subsp. *indica* (V). From all, subspecies enterica (I) is predominant in warm-blooded animals responsible for most *Salmonella* infections in humans and other mammals. In comparison with the other five *Salmonella* subspecies and *S. bongori* which is mainly found in the environment and also in cold-blooded animals (Jajere, 2019).

Salmonella subspecies are also further classified into serovars based on a serotyping scheme developed by Kauffman and White. This scheme involves the identification of three major antigenic determinants: somatic (O), capsular (K), and flagellar (H) which are distinctive surface structures and commonly shared by the species but differ in their chemical constitution (Ibrahim and Morin, 2018). Some serovars like Typhi, Paratyphi C, and Dublin, possess Envelop (VI) surface structures overlaying the O antigen (Romane *et al*., 2012). Currently, around 2610 *Salmonella* serovars are recognized by the Kauffmann-White scheme, with the majority belongs to *S. enterica* about 2587 serovars and the remaining 23 are assigned to *S. bongori* (Nwabor *et al*., 2015).

Even if, the bacteria were discovered by Theobald Smith, it was named after an American pathologist Dr. Daniel Elmer Salmon, who worked with Smith. The Centers for Disease Control and Prevention (CDC) now uses the *Salmonella* nomenclature scheme recommended by the Collaborating Center of the World Health Organization (WHO) (Eng *et al*., 2015).

Currently as of CDC, in subspecies I, names are used for serotypes like Enteritidis, Typhimurium, Typhi, and Choleraesuis, and antigenic formulas are used for serotypes identified after 1966 in subspecies II, IV, and VI, as well as in S. Bongori. The name is generally derived from the geographic place where the serotype was discovered (Brenner *et al.*, 2000).

To demonstrate that, called serotypes are not different species, the serotype name is not italicized and the first letter is capitalized and the name of the genus precedes the term "serotype" or the abbreviation "ser" like *Salmonella* serotype or ser. Typhimurium in the citation of a serotype for the first time. Regarding that, the name may be written with the genus followed immediately by the serotype name like *Salmonella* Typhimurium or S. Typhimurium. Serotype naming by antigenic formula contain subspecies classification (I–VI), then O (somatic) antigens followed by a colon, H (flagellar) antigens (phase 1) followed by a colon, and H antigens (phase 2, if present.) (Brenner *et al*., 2000).

2.4. Virulence factors

Virulence factors play an essential role in establishing *Salmonella* infections. There are various virulence factors including flagella, capsule, plasmids, adhesion systems, and the two, type 3 secretion systems (T3SS) encoded on the *Salmonella* pathogenicity islands (SPI-1 and SPI-2 and other SPIs). Additionally, adhesins, invasins, fimbriae, hemagglutinins, exotoxins, and endotoxins in combination with each other or alone involved in colonization of *Salmonella* to its host through attaching, invading, surviving, and bypassing the host's defense mechanisms such as the gastric acidity, gastrointestinal proteases, and defensins as well as aggressins of the intestinal microbiome (Jajere, 2019).

Salmonella virulence genes are clustered in regions called *Salmonella* pathogenicity islands (SPI) that are spread around the chromosome. These gene clusters are believed to have been acquired by *Salmonella* via horizontal gene transfer from other organisms. This hypothesis is supported by the substantial difference in GC content of the islands when compared to the residual genome and remnants of bacteriophages or transposon insertion sequences that often mark the islands' borders. The acquisition of SPIs may have resulted in a sudden increase in

Salmonella pathogenicity during evolution because they contain several functionally similar genes required for a particular virulence phenotype. Around five SPIs (designated SPI-1 to SPI-5) were previously described On the *Salmonella* chromosome, which is found on centisomes 63, 31, 82, 92, and 25 cs (Van Asten and Van Dijk, 2005).

The SPI-1 is known to encode a type III secretion system, that transfers bacterial proteins such as SptP and SopE of actin-binding proteins into the target cell's cytosol and enables the bacterium to be taken up by the cell. similarly, the SPI-2 locus encodes for a second type III secretion system that is necessary for bacterial survival in both epithelial cells and macrophages of the host. Among 10 open reading frames contained in and encoded by SPI-3, *Salmonella* specific gene called gtC gene is essential for the growth of bacteria in an Mg2+-limited area, such as that found in phagosomes. Bacterial intra-macrophage survival is also facilitated via gene encoded by SPI-4, which also contains a toxin secreting type I secretion system. The SPI-5 harbors 6 genes amongst a gene encoding an effector protein called the SopB gene is included (Van Asten and Van Dijk, 2005).

2.4.1. Type III secretion systems

The Type III secretion systems are specialized virulence devices evolved to direct translocation of bacterial virulence proteins into the host cell cytoplasm. Type III secretion systems have a complex structure made of various structural proteins. That form a needle-like structure in the envelope of the bacteria and consist of two rings that provide a continuous path across the inner and outer membranes, including the peptidoglycan layer. It becomes active upon contact with epithelial cells in the intestine of the infected host and translocates effectors across the host cell plasma membrane (Coburn *et al.*, 2007).

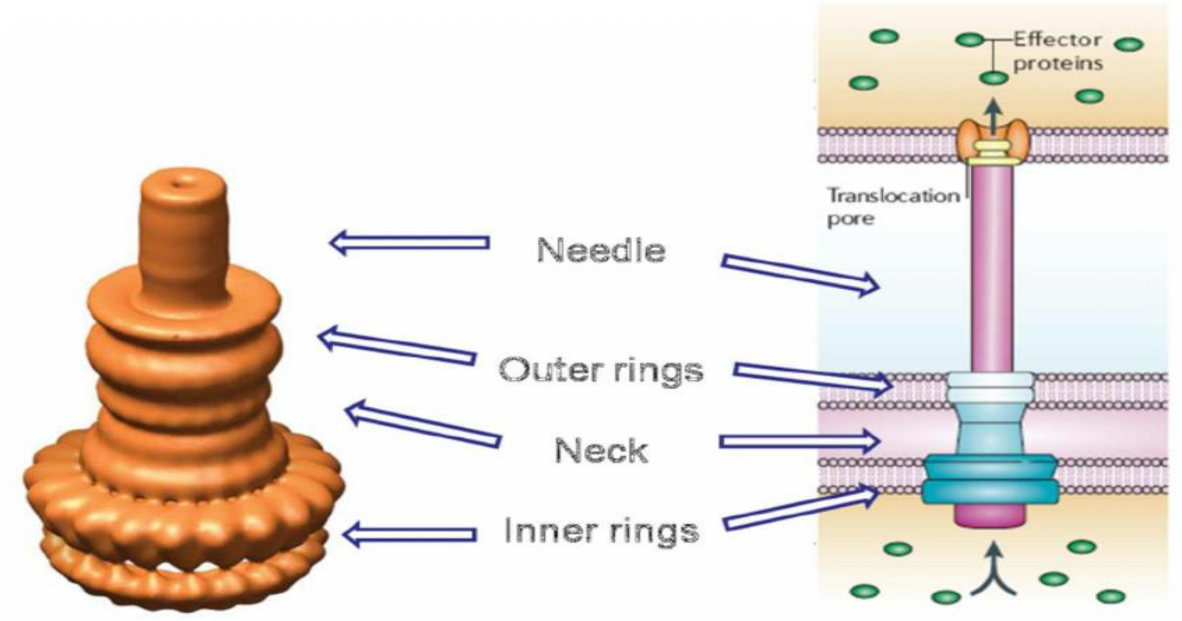

Figure 1: Schematic representation of the specialized virulence devices, T3SS

Source: (Deligios, 2014)

T3SS are encoded by a set of genes forming the Pathogenicity islands to direct injection of effector proteins into host cells. T3SS1 effectors also activate Mitogen-activated Protein Kinase (MAPK) pathways, resulting in pro-inflammatory cytokines generation, the polymorphonuclear leukocytes (PMNs) recruitment, and acute intestinal inflammation development. T3SS2 is present in all subspecies of *S. enterica* but not found in the species *S. bongori*. This system is expressed in response to acidic pH and nutrient limitation intracellularly in the lumen of the SCV. It helps the effectors to be translocated across the membrane of the SCV. These effectors are required for the modulation of the intracellular environment (Ramos-Morales, 2012).

2.4.2. Virulence plasmids

The virulence plasmid of *Salmonella* also known as PSV plasmids, ranges in size from 50 kb - 100kb, and there may be considerable differences exist between plasmids among individual serovars. PSV plasmids contain spv operon region which is a highly conserved region and typical locus of psv and accounts for about 8 bp, it is not present in *S. bongori* and is found in limited distribution to serovars of *S. enterica* subspecies I, II, IIIa, IV, and VII. The *spv*

sequences incorporated in the chromosome of all isolates groups belonging to subspecies II, IIIa, and VII, whereas only some isolates of serovars belonging to subspecies I showed hybridization, in which the *spv* region seemed to be encoded on an F-related plasmid (Fluit, 2005).

This locus harbors one regulatory gene spvR and four structural genes spvABCD, designated as spv RABCD, the spvR gene codes for a transcriptional regulator like LysR that positively regulates independent gene transcription as well as spvABCD operon transcription. A type-three secretion system-2 facilitates the translocation of the SpvB, SpvC, and SpvD proteins to the host cell. SpvB has inhibition functions of actin polymerization, during intracellular host infection by ADP-ribosylation of actin monomers, SpvC is an anti-inflammatory effector which also translocated via T3SS-1, with the function of phosphothreonin-lyase and inhibition of MAP kinases, results in inhibition of immune signaling. SpvD has the inhibitory activity for NF-κB regulated promoters by interfering with nuclear translocation of p65 which results in the downregulation of proinflammatory responses (Silva *et al.*, 2017).

During in-vitro growth of the Bactria, there is no expression of spv genes. But after entry into the host cell including macrophages, its expression began rapidly. the stationary-phase alpha-factor RpoS controls the transcription of the spv genes while mutations in RpoS abolish virulence. The Expression of the spv genes might play a role in the multiplication of intracellular *Salmonella* within the reticuloendothelial system including liver cells and the spleen. Furthermore, other plasmids are likely to contribute to some resistance observed among *Salmonella* serovars (Guiney *et al.*, 1995).

2.4.3. Toxins

Endotoxin is one of the *Salmonella* toxins, which is the lipid portion (lipid A) of the outer membrane lipopolysaccharide (LPS) of *Salmonella*. It is highly associated with life-threatening sepsis and is known to evoke a diversity of biological responses both in vivo and in vitro. The second one of *Salmonella* toxins are exotoxins, which are subdivided into two types: the cytotoxins that can kill the mammalian cell and the enterotoxins. There is a significant difference in the amounts of toxin produced by either serotype (Yalew, 2020).

The *Salmonella* enterotoxin gene (*stn*-gene) is critical in *S.* Typhimurium virulence that encodes for 29 kDa protein. *Salmonella* enterotoxin leads to loss of intestinal fluid as a result of the accumulation of adenosine monophosphate due to activated adenylate cyclase in the enterocyte's cytoplasmic membrane (Gut *et al*., 2018).

2.4.4. Flagella and fimbriae

The flagellum is a long filamentous structure found in motile organisms. *Salmonella* swims in liquid environments and moves on solid surfaces by rotating their flagella. It is one of the important virulence traits that mediate bacterial attachment and invasion. After, oral entry of flagellated strains of *Salmonella* spp. the flagellin enables the organisms to move, attach and colonize the intestinal cell. As a result, a non-motile strain had a lower capability to adhere to cells than the parental strain. The recognition of flagellar protein, by a host cell receptor called Toll-like receptor (TLR)-5 leading to activation of a proinflammatory response and release of the cytokines necessary to initiate the innate and adaptive immune responses. The severe local inflammation activated during the infection helps to confine the bacteria to the intestine and reduce the systemic infection. (Barbosa *et al.*, 2017).

Fimbriae are miscellaneous surface protein structures. That most commonly used in adhesion systems and plays important role in pathogenesis. Furthermore, involves biofilm formation, seroconversion, haemagglutination, cellular invasion as well as macrophage interactions. It is also used as a source of diversity for *Salmonella* serovars due to its expression differentially in each serovar and is an important determinant of host adaptation by *Salmonella* (Yalew, 2020).

2.5. Pathogenesis

A *Salmonella* organism can invade host cells and become an intracellular parasite, after their entry through an oral route with contaminated water or food into the digestive tract. The host first defense mechanisms like the acidity of the stomach and the bile salts of the small intestine, which have a bactericidal effect, face the newly entered *Salmonella*e. However, the acidic conditions in the stomach reduce the infective dose of the bacterium, *Salmonella* has

an adaptive acid-tolerance response. This is due to the presence of sigma factor RpoS on RNA polymerase, which regulates the gene found on the Spv plasmids and triggers the transcription of responsible genes for acid tolerance, and promotes their survival in the low pH milieu of the stomach (Haraga *et al*., 2008).

Once it reaches the small intestine, quick adherence of the bacterium to the intestinal mucosa is required, this involves more than one adhesins such as Agf, Pef, and Lpf. Bacterial-mediated endocytosis helps the bacterial invasion of the target non-phagocytic enterocytes of the intestinal epithelium. which is characterized by the presence of M cells and the absence of mucus-secreting cells overlying the gut-associated lymphatic tissue in the distal small intestine and the upper large bowel. This will allow the bacterium to pass through the lymphoid follicles of the ileum called Peyer's patch, by the involvement of fimbriae (adhesins), for recognition and binding of *Salmonella* to Peyer's patch (Haraga *et al*., 2008; Quinn, 2013).

Similarly, after reaching the intestine the bacteria face stress environment (poor nutrition and antibiotics) that may highly reduce the infective dose. Following adhesion, the entry into the Peyer patch requires the presence of type III secretion systems to inject effector proteins called Ssps and Sops into the target host cell. That triggers the induction of membrane ruffling for bacterial internalization. This results in the alteration of the compartment structure of the host cell. By remodeling the host cellular functions, converting immunity, establishing a survival vacuole, and promoting pathogen proliferation. this permits the intracellular survival and replication of the bacteria within the host cells(Quinn, 2013; Ramos-Morales, 2012).

In general, the interaction of *Salmonella* with host cells triggers various host responses. That is responsible for the induction of intestinal inflammatory responses and systemic illness. Specifically, the identification of bacterial and host proteins that are involved in such processes as the invasion of epithelial cells, stimulation and repression of signaling cascades, sensing of the intracellular environment, and establishment of a niche for intracellular replication have a contribution to the formation of the complex pathogenesis of this bacterium (Haraga *et al*., 2008).

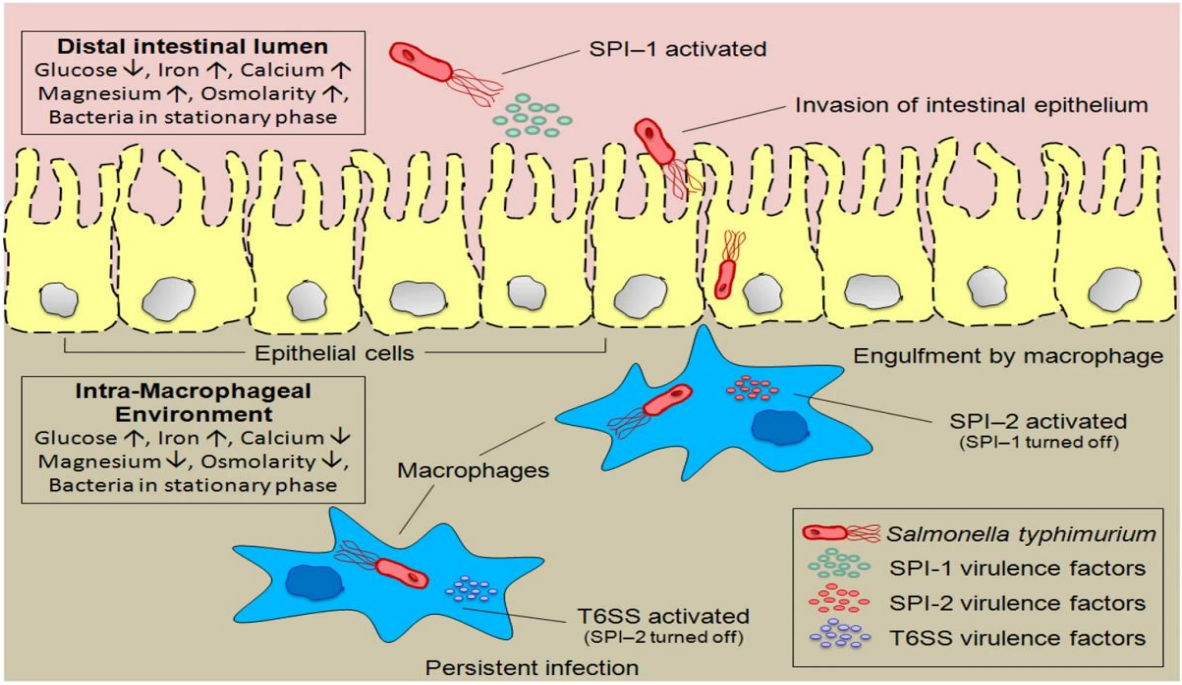

Figure 2: A sequential activation of SPI-1, SPI-2 &T6SS during chronic *Salmonella* infection pathogenesis

Source: (Das *et al*., 2013).

2.6. Clinical features of Salmonellosis

Salmonella is a highly competent organism and can cause disease in both humans and animals. Enteric fever is one of the disease patterns in human *Salmonella* infection caused by serotypes *S*. Typhi and *S*. Paratyphi A, B, and C, which results in typhoidal fever as well as paratyphoid fever respectively. That characterized by 3 weeks of incubation period on average, a sustainable gradual onset of fever ranges from 39–40°C. The symptoms include headache, abdominal pain, and diarrhea more commonly in children. Constipation predominantly immunocompromised patients. Besides that infected patients may also develop myalgia, bradycardia, hepatomegaly (enlarged liver), splenomegaly (enlarged spleen), and rose spots on their chest and abdomen (Eng *et al*., 2015).

Nontyphoidal Salmonellosis is caused by 150 *Salmonella* serotypes including *Salmonella* Typhimurium and *Salmonella* Enteritidis to result in Gastroenteritis or enterocolitis. It is an inflammatory condition of the gastrointestinal tract characterized by massive neutrophil

recruitment across the intestinal epithelium. This infection was kept in the terminal ileum and colon in immunocompetent patients. Highly affecting Infants, young children, elderly people, and immunocompromised patients. Characterized by self-limiting Symptoms includes vomiting, non-bloody diarrhea, nausea, headache, fever, abdominal cramps, and myalgias. Which lasts only for 10 days or less, with a shorter incubation period of about 1 day and Gastrointestinal complications include cholecystitis, pancreatitis, and appendicitis (Gal-Mor *et al*., 2014).

Bacteremia is another feature of salmonellosis; it is a serious condition in which bacteria enter the bloodstream after passing through the intestinal barrier. It has been associated with highly invasive serotypes includes *S*. Typhi, *S*. Paratyphi, *S*. Choleraesuis, and *S.* Dublin that result in high fever without the formation of rose spots as observed in patients with enteric fever. But it is relatively associated in low proportion with *S.* Typhimurium, *S*. Enteritidis, and *S*. Heidelberg infection. Both age extremes and immunocompromised are populations at risk for the development of extraintestinal invasive salmonellosis. In cases of severe bacteremia septic shock, meningitis, septic arthritis, and osteomyelitis could be concomitant with the occurrence of a high mortality rate due to the triggered immune response (Pui *et al*., 2011; Su, 2014).

Chronic carrier state is the status of shedding of bacteria in stools for more than a year after the acute stage of *Salmonella* infection. Since human beings are the source of *Salmonella* Typhoid and Paratyphoid infection, the transmission will be through common transmission routes like the consumption of carriers feces contaminated water, and food (Wibisono *et al.*, 2020).

Individuals, who work in food-related industries, may highly contribute to the spread of Salmonellosis to a human being. On average, nontyphoidal serotypes persist in the gastrointestinal tract from 6 weeks to 3 months, depending on the serotypes. Only about 0.1% of nontyphoidal *Salmonella* cases are shed in stool samples for periods exceeding 1 year. About 2 to 5% of untreated typhoid infections result in a chronic carrier state. Up to 10% of untreated convalescent typhoid cases will excrete *S*. Typhi in feces for 1 to 3 months and between 1 and 4% become chronic carriers excreting the microorganism for more than one year (Pui *et al*., 2011).

Animals also shed *Salmonella* in their feces after infection and become the main source of NTS in human infection through the contaminated environment, food, and water. The shedding of non-typhoidal salmonellosis in clinically affected animals has a higher prevalence in comparison with apparently healthy animals, but the duration of shedding is for long periods in both cases (Hoelzer *et al*., 2011).

2.7. Route of transmission

Contaminated foods and water are the major sources of *Salmonella* infection in humans. As, *Salmonella* lives ubiquitously in nature and lives in the intestinal tract of animals, birds, and humans. It may shade in their feces and leads to the contamination of the environment and foods. It can survive well in a variety of foods which become the most common vehicles of salmonellosis. The contamination of fruits and vegetables can be occurred before or after harvest that becomes a source of human salmonellosis. And also contaminated seeds, irrigation water, and flooding have a contribution to the transmission (Shekhar, 2018).

From the food of animal origin, Poultry products are frequently the potential source of infection. Consumers can be infected with *Salmonella* while they eat raw or undercooked food from the animal origin for example *S*. Enteritidis silently infects the ovaries of apparently healthy hens and contaminates the eggs before the shells are formed. Pork, Beef, and dairy products are also important vehicles in the transmission of salmonellosis to humans (Shekhar, 2018).

2.8. Epidemiology

salmonellosis is recognized worldwide but seems most prominent in intensive animal husbandry areas, particularly in poultry and pigs (Weldo, 2014). *Salmonella* is mainly intestinal bacteria, therefore the intestinal tract of several animals as well as birds, especially poultry and swine are the primary reservoir for salmonellosis. Then the bacterium contaminates a wide spectrum of places through insects and other creatures that carry the organism from fecal excrete of the reservoir host (Nwabor *et al*., 2015). So they are widely

distributed in the environment and can be found in farm effluents, human sewage, and any object that has been contaminated with feces (Weldo, 2014).

The epidemiology of salmonellosis is complicated by the fact that the presence of many serovars (over 2,500 different serotypes) with different reservoirs and diverse spatial incidences. Prevalence and incidence of disease vary greatly between different geographical areas. And highly dependent on environmental factors such as climate, population density, food harvesting, and processing technologies, as well as host factors like the habit of the consumer (Pal *et al*., 2020). Changes in food intake, processing, and distribution have increased the number of multi-state outbreaks related to fresh and processed foods (Weldo, 2014).

As per the WHO Global Salm-Surv, *S*. Enteritidis was by far the most common serotype recorded from humans worldwide between 2000 and 2002. It accounted for 65% of all isolates in 2002, followed by 12% of *S*. Typhimurium and 4% of *S*. Newport (Uro, 2019).

In non-human isolates, *S*. Typhimurium was the most widely identified serotype in all three years with 17 percent of isolates in 2002. this is followed by *S*. Heidelberg and *S*. Enteritidis with 11 and 9 percent respectively. During the three-year study cycle, *S*. Enteritidis, *S*. Typhimurium, and *S*. Typhi were among the 15 most prevalent human serotypes in all regions of the world. *S*. Agona, *S*. Infantis, *S*. Montevideo, *S*. Saintpaul, *S*. Hadar, *S*. Mbandaka, *S*. Newport, *S*. Thompson, *S*. Heidelberg and, *S*. Virchow were also widespread. In Africa in 2002, *S*. Enteritidis and *S*. Typhimurium were each reported from approximately one-fourth of isolates from humans (Uro, 2019).

In certain countries in Central and South America, Africa, and Asia, fowl typhoid and pullorum disease are usual. Many other countries with mature poultry economies, such as the United States, Canada, New Zealand, Australia, Japan, and the majority of European countries, have eradicated these diseases from commercial poultry. however, S. Gallinarum and S. Pullorum can persist in backyard flocks, game birds, and wild birds even in these countries (Shivaprasad, 2019).

2.8.1. Host range

The genus *Salmonella* has a wide range of hosts to cause disease in a variety of warm- and cold-blooded animals. A wide variety of hosts and pathogenicity is due to past within-host evolution invertebrate organisms that changed ancestral pathogenesis mechanisms. Point mutations, new genes acquired via horizontal gene transfer (HGT), deletions, and genomic rearrangements are involved in the variation that arises throughout infection. Beneficial mutations become more common within the host and, if they can be passed on to subsequent hosts, they may become fixed in the population (Tanner and Kingsley, 2018).

In host-pathogen interactions irrespective of the degree of Pathogenicity, Pathogens referred to as generalists can infect a large range of host species whereas others have a high association of isolation with a particular host, causing systemic infection referred to as specialized or host restricted strains that create an intimate connection to only one host species. The majority of pathogens could infect multiple hosts. Genericism is favored by genetic variation and ecological potential for cross-species transmission. The evolutionary process that leads to specialism has long been debated (Baumler and Fang, 2013).

Serovar host specificity or serovar host adaptation is a phenomenon in which a particular mechanism making a serovar virulent for one particular animal species could make the same serovar less or even avirulent for another animal species. The host specificity and adaptation of particular pathogenic *Salmonella* depends on the specific ability of serovar's to adapt to the environment of their hosts. This is regulated by many microbial characteristics and infective dose as well as host factors (Jajere, 2019).

Under some conditions, all animal species are susceptible to different *Salmonella* serovars, but not all are clinically affected to the same extent. Specific serovars affecting specific animal species are linked to clinical severity. Scientifically, all pathogens should be classified as host-adapted pathogens, regardless of epidemiological variations between related microorganisms that indicate some having a single susceptible host and others having a larger number (Evangelopoulou *et al*., 2013).

Therefore, the pathogenic characteristics of the particular serovar and epidemiological observation in a susceptible host should be established to determine the significance of 'host specificity-host adaptation' in *Salmonella* infections. Those attempts to define the pathogenicity predictors of *Salmonella* serovars that cause infections in humans and animals have resulted in three large classes of serovars (Evangelopoulou *et al*., 2013).

Serovars are being divided into three classes based on their host specificity. The majority of serovars, such as *S.* Typhimurium and *S*. Enteritidis, are generalists that can infect a wide variety of host species. The second category of serovars is host-adapted serovars, which normally cause disease in one host species but can accidentally infect other hosts, including humans, like *S*. Choleraesuis (swine adapted), *S*. Dublin (bovine adapted), and *S*. Abortusovis (sheep adapted), are examples of such serovars (Cohen *et al*., 2021).

The third group of serovars are those that are strictly host-specific (or host-restricted) so can only infect one host species, such as *S*. Typhi and *S*. Paratyphi A, which only infect humans and higher primates and cause a systemic life-threatening enteric fever. Likewise, other serovars, *S*. Typhisuis in pig, *S.* Abortusequi in equine, *S*. Gallinarum, and *S*. Pullorum, have been adapted for the avian host, causing the systemic invasive disease called fowl typhoid and pullorum disease respectively are highly host-adapted or host-restricted (HR) serovars and strictly associated with one very specific host (Cohen *et al*., 2021). The non-host adapted serovars of *Salmonella* are more responsible for the overwhelming incident of human salmonellosis and cause infection in many other animal species. Even if they are less virulent, they have the most greater epidemiological importance as compared to the host-adapted one (Nwabor *et al*., 2015).

2.9. Status in Ethiopia

In Ethiopia, the occurrence and the status of food-borne *Salmonella* infection are not well known. The origin of human infection and the risk of contaminated food from the animal source has not been well studied and reported. Almost all over the country population’s habit for the consumption of raw and undercooked animal products and the presence of carrier animals considerably implies the risk of getting *Salmonella* infection and MDR *Salmonella* from animal products (Tadesse and Gebremedhin, 2015).

The most frequently isolated *Salmonella* serovars in the country were *S*. Dublin, *S*. Infantis, and *S*. Saintpaul from beef, small ruminant, and camel meat, respectively. Likewise, *S*. Newport and *S*. Braenderup in pork and chicken meat respectively. Among them, *S*. Braenderup, *S*. Newport, *S*. Dublin, *S*. Infantis, *S*. Saintpaul, and *S*. Typhimurium are prevalent in human clinical samples. And also *S*. Anatum, *S*. Newport, and *S*. Dublin are mostly isolated in samples collected from personnel working in abattoirs or markets. As Tadesse and Gebremedhin report; revealing that the isolates from the market, milk, and meat were MDR serovars with some resistant strains for drugs not commonly used in the veterinary sector (Tadesse and Gebremedhin, 2015).

2.10. Diagnosis of *Salmonella*

The conventional detection method of Salmonella

Cultivation procedures are a traditional method for isolation of *Salmonella* spp. and are considered as a gold standard method. That involves steps like Pre-enrichment, followed by selective enrichment and plating onto selective agars as well as the biochemical and serological confirmation of the organisms. The pre-enrichment step is used to recover *Salmonella* from the sample by inhibiting the growth of other competing flora, this step uses a nutritious nonselective medium with the formula to increase the sensitivity of *Salmonella* detection. The most commonly used pre-enrichment Media are BPW and lactose broth (Lee *et al*., 2015; Schultz *et al*., 2012).

Selective enrichment is the second step in detecting and isolating *Salmonella* spp., this also works by selectively grow *Salmonella* and inhibit the growth of other bacteria due to the presence of inhibitory compositions in the medium. Like bile salts, brilliant green, thiosulphate, deoxycholate, malachite green, novobiocin, tetrathionate, cycloheximide, nitrofurantoin, and sulphacetamide. The most commonly used Selective enrichment media are Rappaport Vassiliadis (RV) medium and tetrathionate (TT) broth (Lee *et al*., 2015; Schultz *et al*., 2012).

The plating out on solid selective and differential media is the third step to isolate presumptive positive *Salmonella* colonies while suppressing other bacterial growth. The

most commonly used plating media are Xylose-lysine-deoxycholate agar (XLD), brilliant green agar (BGA), and Hektoen enteric (HE). Then cultures giving presumptive *Salmonella* colonies were subjected to biochemical tests for further identification of isolates. This is performed by streaked on different media such as triple sugar iron agar for glucose fermentation test, lysine iron agar for testing of lysine decarboxylase reaction, followed by urease test and additional tests for urease-negative culture (Lee *et al.*, 2015).

Serological and Immunology-based assays

This type of assay for detection of *Salmonella* spp. employs a specific antibody that binds to somatic or flagella antigens (Di Febo *et al*., 2019). It is a rapid test and able to detect non-cultivable organisms with high specificity but has some limitations of cross-reactivity with closely related antigens and is less sensitive with antigen variation (Lee *et al*., 2015). This Includes the rapid serum agglutination test, whole blood agglutination test (WBT), micro-antiglobulin or microagglutination, the standard tube agglutination test, and recently, Enzyme-linked immunosorbent assay (both indirect and competitive ELISA), latex agglutination tests, immunodiffusion, and Immunochromatography. The use of antibodies especially IgG which can persist for many weeks after infection of *Salmonella* and the response of the tests in the absence of infection make the assays are more advantageous over bacteriological examination (Hafez, 2001).

Immunohistochemistry (IHC)

Immunohistochemistry is a highly sensitive and specific diagnostic technique used for salmonellosis infection in animals. It is a combination of immunologic reactions and chemical reactions with photonic microscopy. this type of test has an advantage over bacteriology due it allows the antigen localization and detection within lesions or tissues even as the organism is dead. Based on those qualities IHC immediately increases the accuracy of the diagnosis and brief the pathogenesis of infection (Mshelbwala *et al*., 2018).

2.10.4. Nucleic acid-based assays

The nucleic acid-based assays are the most sensitive and specific assay. That involves detection and characterization procedures using DNA/RNA methods (Barken et al., 2007) and also can rapidly recover *Salmonella* directly from the sample without pure culture by using a specific sequence of the nucleic acid of an organism. Currently, Nucleic acid-based assay includes polymerase chain reaction in a real-time format, endpoint PCR with microfluidics and array technologies, and integrated platforms in which nucleic acid extraction, amplification, and analysis are performed in a single step. Isothermal amplification is also a recently known technique for pathogen detection which does not require thermal cycling(Amjad, 2020). This variant PCR requires the use of DNA probes that are labeled by fluorescent and optical fluorescence signal detectors. It also requires increased costs and well-trained laboratory personnel (Xi, 2018).

2.11.Salmonellosis in Chickens

A large number of *Salmonella* serotypes are capable of colonizing and infecting chickens including both broiler and egg layers. *Salmonella* Gallinarum and *Salmonella* Pullorum are most commonly associated with poultry salmonellosis to cause the most important bacterial disease called Fowl typhoid (FT) and pullorum disease (PD) in poultry. Both of these diseases show similar clinical signs such as depression, weakness, somnolence, loss of appetite, drooping wings, huddling, dehydration, and ruffled feathers with an incubation period ranges from 4 up to 6 days. Morbidity ranges from 10 to 100 percent, with mortality reaching 100 percent in stressed or immunocompromised flocks. This is a serious concern in all types of young and adult chickens. The bacterium is resistant to a normal environment and can live for months, although it is susceptible to common disinfectants (Shoaib *et al.*, 2017).

The infection associated with *S.* Enteritidis and *S.* Typhimurium serotypes are characterized by less clinical severe forms of illness and the animals usually become asymptomatic carriers, but in young chicks, acute outbreaks may occur which exhibiting clinical disease accompanied by high mortality rates (Clemente *et al*., 2014).

The susceptibility of chickens to *Salmonella* infection was determined based on different factors. This includes the age of birds, *Salmonella* serotype and initial challenge dose level, stress including environmental, transport, and overt or subclinical disease, the presence of feed additives, such as antimicrobials, and anticoccidials. Additionally, survival through low pH of the stomach, competition with gut microflora, presence of a compatible colonization site, and host genetic background (Clemente *et al*., 2014).

2.11.1. Transmission of Salmonellosis in chickens

There is two transmission route of *Salmonella* infection in chickens such as vertical and horizontal transmissions. Vertical transmission is when chicks get an infection from infected parent stock through the hatchery. In which one or more eggs, originating from infected breeders, are contaminated and cause some chickens to be infected at hatch. While the horizontal transmission is when chickens get an infection from feed, water, hatcheries, in sexing, cloacal infection, transport equipment, and contaminated bedding material, unclean facilities, and vectors, such as humans, birds, insects, and rodents in poultry farms (Foley *et al*., 2008).

The fecal-oral route is also another means of transmission of *Salmonella* from infected chickens to susceptible flock-mates when feces from infected chickens or faecally contaminated substances are picked by healthy ones from the environment. In general, the introduction of the organism into a flock and transmission between flocks is complicated, which means *Salmonella* infection originates from another flock in another pen or a previous flock in the same pen (Heres *et al*., 2004).

Fowl typhoid and pullorum disease are horizontally transmitted orally by ingestion of contaminated food and water or by cannibalism with *Salmonella* Gallinarum and *Salmonella* Pullorum. The causative agent also enters via the respiratory tract and wounds. *Salmonella* Pullorum has a vertical transmission route and has been detected in eggs. This phenomenon is characterized by the infected some animal may become a carrier for a long time without showing the clinical signs, but still transmit it to their progeny in eggs. Even if *Salmonella* Gallinarum also detectable in eggs its vertical transmission significance is not clear (Shivaprasad, 2019).

The survivance of *Salmonella* Gallinarum and *Salmonella* Pullorum in the normal condition ranges from several months up to several years in the environment. However, exposure to sunlight and high temperatures may affect their survival period in the environment. Mechanical or biological vectors such as insects, red mites (*Dermanyssus gallinae*), wild birds, and mammals are involved in spreading fowl typhoid and may maintain these bacteria for several months (Shivaprasad, 2019).

Likewise, *Salmonella* Enteritidis also has a horizontal transmission and contributes to vertical transmission by infecting the chicken reproductive system via migrating from cloaca to the reproductive organ. The systemic infection of *Salmonella* Enteritidis in mother birds results in transovarian infection includes ovary infection and egg production in the oviduct (Wibisono *et al.*, 2020).

2.11.2. Prevention and Control Method

Salmonella prevention and control can be achieved by combining Good Agricultural Practices and Hazard Analysis Critical Control Point (HACCP) principles with other measures including vaccination, competitive exclusion, use of organic acids, culling, and product diversion to processing. Antimicrobial drugs should not be used to treat *Salmonella* infection in chickens because their efficiency is limited, they can hide the illness during sampling, they can leave residues in meat and eggs, and they can contribute to antimicrobial resistance. Antimicrobials may potentially disrupt natural gut flora, increasing the risk of colonization (World Organisation for Animal Health (OIE), 2019).

Prevention strategies should be implemented from the farm to the fork to prevent *Salmonella* transmission to humans. This includes the whole meat as well as the egg production chain and subsequent storage and handling. To put it differently, the control measures should emphasize the pre-harvest (at the farm), the harvest (catching and shipping), and the post-harvest (at the abattoir, the market, and in the consumer's kitchen or restaurant) phases (Van Immerseel *et al.*, 2009).

Hygiene and Biosecurity Measures

To control *Salmonella* infections, general hygienic and biosecurity measures are critical because when the overall biosecurity plan fails, all other measures become ineffective. The introduction of *Salmonella*-free animals is a critical step in preventing infections. followed by a proper cleaning and disinfection program to reducing the number of micro-organisms in the poultry house, feed and water decontamination as well as insect and rodent control program (Van Immerseel *et al.*, 2009).

Vaccination of Chickens

Chicken vaccination, along with other intervention methods, is a key strategy now being utilized to reduce *Salmonella* levels in poultry flocks, which will eventually lead to a lower incidence of human *Salmonella* infections. Vaccination, actuate the host immune system to reduce, rather than controlling *Salmonella* spp. associated with poultry upon infection. Vaccinations against young chicks are not as effective during the first week as their immune system has not developed. due to this vaccination tackle the unique challenge for broiler chickens whose life is as short as 5-6 weeks. Therefore, the broiler industry can be required to use vaccines that can provide protective immunity to the progeny of breeder flocks and competitive products of exclusion to the young chicks. Vaccines for *Salmonella* have been tested and categorized into live-attenuated, inactivated, and subunit vaccines. The vaccine should ideally be safe, cross-protective, well-defined, and induce cell immunity that is thought important in controlling *Salmonella* infections in all species (Desin *et al*., 2013).

2.12. Public Health and Economical Importance

The wide host range of *Salmonella* (*S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg) serotypes have worldwide distribution and significant economic and public health importance. The transmission of *Salmonella* can be both from animal to human or human to animals. Humans can get *Salmonella* infection, mostly through contaminated food and water, veterinarians and farm workers may get the infection directly from infected live chickens on the farm. Person to person transmission may also occur, indirect infection from infected or

contaminated food sellers, from infected workers in a chicken farm and within hospitals (Salem *et al*., 2016; Wibisono *et al*., 2020)

Salmonellosis associated with contaminated meats and poultry is a common human intestinal disorder. Primarily Infections with *Salmonella* in food-producing animals present a serious public health concern because food products of animal origin are considered to be a significant source of human infection. The most common sources of infection are eggs and meat from poultry and other food animal species. Milk and dairy products have also been associated with outbreaks of salmonellosis in people. In addition, contamination of fruit and vegetables by infected water may also be a source of infection (Yalew, 2020)

Human salmonellosis is associated with *Salmonella* in poultry. The distribution of different serotypes is unequal in humans and poultry, and the different serotypes in poultry are probably not equally pathogenic for humans. Nevertheless, considering the relationship between humans and poultry, all phases in the poultry production chain, including the farm, should seriously control *Salmonella* (Heres *et al*., 2004).

Salmonellosis has a huge economic impact on one country. This is related to the costs of clinical disease, which include the costs of mortality, laboratory diagnosis, treatment, cleansing, and disinfection, as well as control and prevention. In livestock production, the loss includes Feed efficiency, lower weight gains, and mortality due to Salmonellosis. In the case of Human Salmonellosis, the financial costs are not only related to the investigation, treatment, and prevention of human illness, but they can also influence the production chain. Poultry products have usually been the leading cause of Salmonellosis in many developing countries, and even in affluent countries, it is one of the most seriously considered food concerns in chicken meat. *Salmonella* contamination in poultry products can occur at any time in the food supply chain, including manufacturing, processing, distribution, retail marketing, handling, and preparation (Uro, 2019). Besides the expenses of morbidity and mortality in humans and animals, trade restrictions and the disposal of contaminated food are significant socioeconomic issues associated with bacteria. (Tauxe *et al*., 2010).

2.13. Antimicrobial Resistance

Antibiotics are antimicrobial compounds, that can suppress the growth of microorganisms or kill them. they are broadly used for treatment in human and animal bacterial infections. Excessive use of antibiotics has resulted in a significant increment of antibiotic excretion and environmental release rates, this leads to the spread of antibiotic-resistant bacterial strains. Additionally, the Antibiotic-resistant phenomenon aggravated with the use of a wide range of antibiotics to enhance food-animal growth. The overuse and misuse of antibiotics accelerate the development of Antibiotic-Resistant bacteria and Antibiotic-Resistant genes in the environment, thereby increasing the risk of transmission of environmentally resistant bacteria to humans. Which are a global concern and a considerable human health problem. Resistant bacteria result in much more complicated illness that requires difficult treatment, more expensive drugs as well as drugs with high toxicity (Serwecińska, 2020).

The emergence of antimicrobial resistance in *Salmonella* was first reported in the early 1960s for a single antibiotic called chloramphenicol. Now it has become a serious health problem worldwide with the report of increased incidence of multi-drug resistance *Salmonella* isolates in many countries (Saha *et al*., 2020).

Several mechanisms (biochemical and genetic mechanisms) can be attributed to *Salmonella* species multi-antimicrobial resistance (MAR), including target site modification; for instance, in fluoroquinolone resistance, there is a mutation in DNA gyrase and topoisomerase IV. Degradation of the antibiotic by hydrolysis in case of lactamases resistance and modification of the drug to an inactive form in case of aminoglycoside resistance. Additionally, efflux pump overexpression, cell envelope mutation, porins down-regulation, and lipopolysaccharide component enhancement over the outer membrane mediates the reduction in effective intracellular concentration. This prevents the antibiotics from accessing their target. quorum sensing and biofilm formation are also other mechanisms that contribute to resistance (Martins *et al*., 2011). Antimicrobial resistance factors can be acquired and disseminate within *Salmonella* species and between other bacteria by transferring mobile genetic elements such as virulence encoding plasmid, transposons, and integrons via Horizontal and vertical gene transfer (Akinyemi and Ajoseh, 2017).

An antimicrobial agent such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole is used as the traditional first-line treatment for *Salmonella* infections. *Salmonella* spp. referred as multi-drug resistant, when it resists towards the above-listed antimicrobials. This is mainly elevated by the use of antibiotics in animal feed to promote the growth of livestock, and in veterinary medicine to treat bacterial infections in those animals. This results in a high risk of MAR *Salmonella* strains transmission from animals to humans through the ingestion of contaminated food or water with the animals' feces, direct contact, or the consumption of infected animal origin food (Threlfall, 2002).

3. MATERIALS AND METHODS

3.1. Study area and period

This cross-sectional study was conducted between November 2020 and July 2021 in and around Adama town, central Ethiopia. Adama town officially known as Adama, and is a city found in Oromia region, central Ethiopia. Adama is an East Shewa Zone town. It is found about 60 miles Southeast from Addis Ababa. The city is coordinated between 8° 30' 52 1172" N and 39° 16' 9.3252" E latitude and longitude, respectively on GPS (https://www.latlong.net/place/adama-oromia-ethiopia-22095.html). The town lies 1623 meters above sea level. The climate of Adama town is categorized as Hot semi-arid *climate* (Bsh) based on the Köppen-Geiger climate classification system. Furthermore, there is very little rain throughout the year ranging from 400 mm to 800 mm. The area has also an average annual temperature of 20.7 °C and annual precipitation amounts to 371 mm (Climate-Data.org, 2020). The town covers approximately 9799 km^2 area which is about 16% of the total area coverage of Oromia region (Figure 3**)**. Adama woreda is one of the districts found in East Shewa and also known as Adama zuriya (the rural kebeles surrounding Adama town) which lies on an elevation from 900 to 2400 m above sea level. The area is suitable for the high production potential of crops and livestock. The livestock population in this area is estimated as 103,440 cattle, 45,554 sheep, 54,112 goats, 44,000 donkeys, 780 horses, 410 Mule, 515 camel, 87,341 Poultry, and 480 modern Beehives, 2240 transitional Beehive and 3914 traditional Beehives (Gebregziabhear, 2018). In Adama and Adama woreda, urban and peri-urban small-scale intensive poultry production systems, as well as the medium and large-scale intensive poultry production systems, are mainly found and practiced. The Broiler production is highly concentrated in Adama, Modjo, and Bishoftu; while Bishoftu is known additionally for rearing pullets and Addis Ababa mainly by egg production (FAO, 2019). The poultry meat supply chain in the small-scale and medium-scale farms found in Adama town and around Adama is slaughtering at their houses and selling to the supermarket.

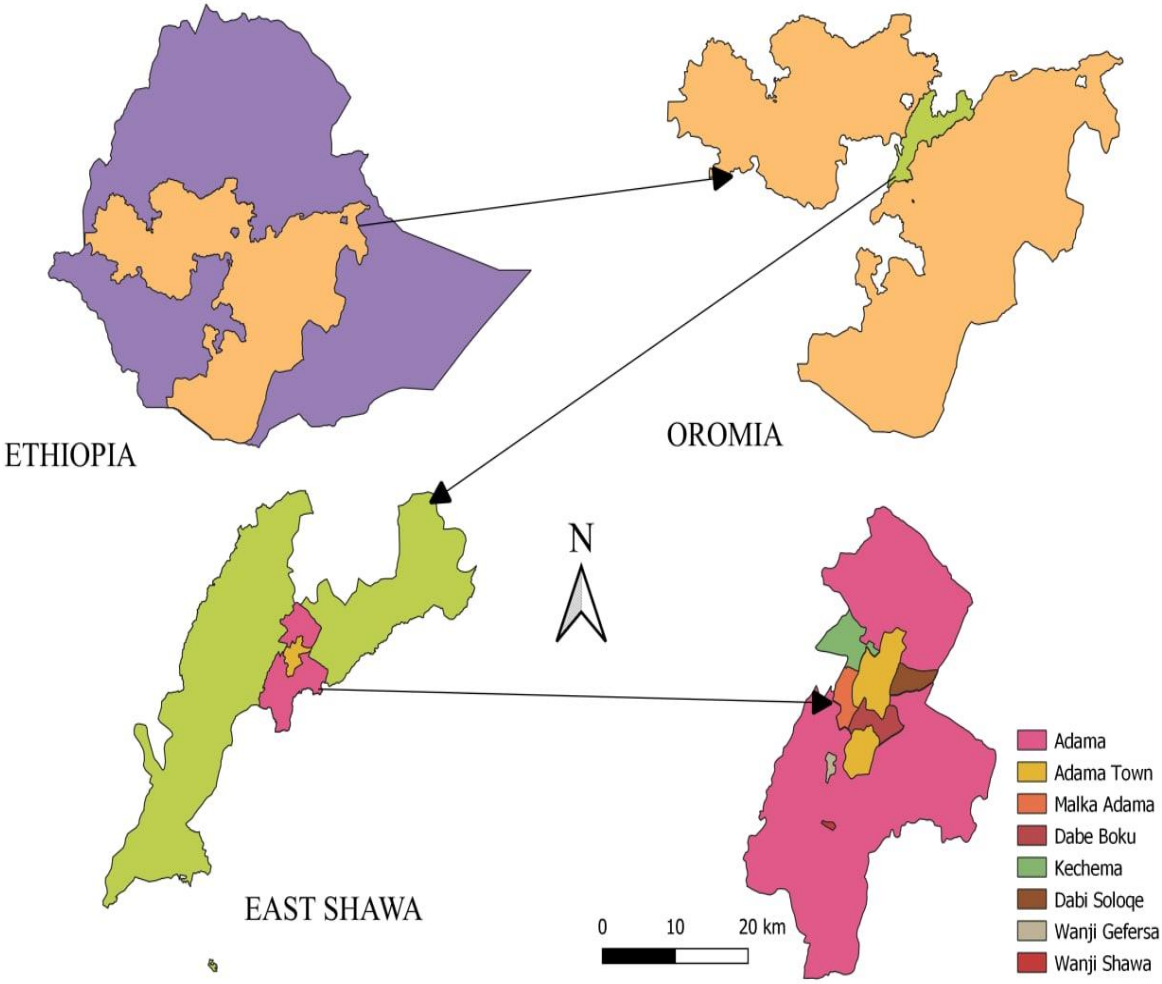

Figure 3: Map of the study area

3.2. Study population and Sample type

The target population consisted of apparently healthy chickens within the selected small scale (500-1000 birds per farm) and medium scale (>1000 birds per farm) (FAO, 2019) broiler chicken farms found in and around Adama, Central Ethiopia. The chickens in the farms were mainly Sasso breeds and Cobb-500, having an age group categorized as $\leq$ 2 months and above two months (Eguale, 2018). All of the selected farms involved in the current study were applying the intensive management system with a deep litter system; they kept their chickens indoors, use modern feeding troughs and watering troughs, and light bulbs. The type of samples was cloacal swab from the chicken, fresh fecal dropping, litter sample from the chicken house, feed sample from chicken feed, and a water sample from chicken drinking water.

3.3. Study design

A cross-sectional study was conducted between November 2020 and July 2021 in and around Adama town, central Ethiopia to isolate *Salmonella* from chickens and their environment's, molecular detection and confirmation of the involved *Salmonella* serotype in the farms and to determine the antimicrobial susceptibility profile for the isolates of *Salmonella* from broiler chickens and their surroundings.

3.4. Sample size

The sample size was determined using the formula described by Thrusfield, (2018) in a 95% confidence interval.

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

Where n = required sample size,

1.96 = the value of Z at 95% confidence interval,

P = expected prevalence which was (0.288) from the previous study on the prevalence of *Salmonella* in poultry conducted on Adama town by Dagnew *et al.* (2020), and

d = desired absolute precision.

Accordingly, the calculated sample size was 315.

3.5. Sampling technique

Six broiler chicken farms (five small-scale and one medium-scale farms) were selected with purposive selection based on the production system, farm availability, proximity to the transportation, and willingness of the owners. Poultry farm lists were obtained from Urban and District Agricultural Offices to get information about available poultry farms and their locations. Three of the farms were located in 3 different kebeles within Adama town, out of the three two farms had 1000 birds and one farm had 600 birds. The remaining three were found in different kebeles around Adama town (Adama woreda). Each of the three farms had a different number of birds: such as 1600, 600, and 500 chickens per respective farm. A minimum of 5% of live birds from each selected farm was selected by applying the simple random sampling technique in which every chicken in the population has an even chance and likelihood of being selected in the sample.

3.6. Sample Collection and Transportation

A total of 380 samples (260 cloacal swab, 30 fecal droppings, 30 litter samples, 30 feed samples, and 30 water samples) were collected from selected 5 small scale broiler farms found in Dabi soloke, Dabe Boku, Melka Adama, Wenji Shewa, and Wenji gefersa kebeles and 1 medium scale broiler farms found in Kechema kebele (Table 1). The chicken cloacal swabs were taken aseptically with sterile, moistened cotton swabs in 10 ml of sterile buffered peptone water. Then it was placed in a screw-capped test tube containing 9 ml of transport media, Buffered Peptone Water (BPW).

The pooled fresh fecal droppings of a minimal 5g were collected from different corners in each poultry house by using clean disposable gloves. Furthermore, a pooled litter samples

weighing 5g each from different sides on the floor of a poultry house were collected by using sterile gloves (Lu *et al*., 2003). Likewise, 5g of pooled feed samples from different feed troughs placed in different corners were also collected in each farm. Then the collected samples were transferred into a sterile vial containing 45 ml of BPW. Similarly, pooled water swabs were collected from different water troughs of each farm and placed in a screw-capped test tube containing 9 ml of BPW.

All the collected samples were properly labeled according to the type of sample, date of sampling, and code of the specific farm. Then, the collected samples were transported to the microbiology laboratory of the College of Veterinary Medicine and Agriculture, Addis Ababa University through an icebox containing ice packs. Upon arrival, the sample was incubated at 37ºC for 24 hours.

Table 1: Summary of the collected sample

Type of sample	No of the collected sample		Total
	In Adama town	Around Adama town	
Cloacal swab	125	135	260
Fecal	15	15	30
Litter	15	15	30
Feed	15	15	30
Water sample	15	15	30
Total	**185**	**195**	**380**

3.7. Sample processing

3.7.1. Isolation and identification

The International Organization for Standardization guideline technique (ISO 6579:2002(E), 2008) and the Global Salmonella Survey of WHO guidelines (WHO, 2010) were employed for the isolation and identification of *Salmonella* organisms. The included steps were the primary enrichment or pre-enrichment in a non-selective liquid medium, secondary

enrichment in selective liquid media, plating out on selective and final confirmation by biochemical and molecular detection (Annex II). All the bacteriological medias were prepared based on to the manufacturer's instruction (Annex IV).

Pre-enrichment

The 5g of each pooled fecal, feed, and litter sample were pre-enriched in 45 ml of buffer peptone water (SRL, BM020, India), in (1:9) ratio then incubated for 24 hours at 37 ºC. Similarly, the cloacal swabs samples as well as water swabs samples were also pre-enriched in 9 ml of BPW and incubated overnight with the same temperature.

Selective enrichment

Following pre-enrichment, about 0.5 ml (500 µl) of the cultured pre-enrichment was transferred to 10 ml of selective enrichment Rappaport-Vassiliadis soy peptone (RVS) broth (HiMedia, M880, India) and incubated at 42 ºC for 24 hours.

Plating on selective media

A loop-full of cultured RV suspensions were streaked onto the surface of selective agar, Xylose lysine deoxycholate (XLD) agar (SRL, XM011, India), and incubated at 37 ^{0}C for 24-48 hours. Then the plates were observed for black-centered pink colony morphology typical of *Salmonella* (Annex VI). The five *Salmonella* suspected, pink with black centered colonies(OIE, 2018) was then taken and plated out onto nutrient agar(SRL, India) to undertake further biochemical tests for typical *Salmonella* (ISO 6579:2002(E), 2008).

3.7.2. Biochemical Examinations

The suspected *Salmonella* colonies were further tested on different six biochemical testes (Annex VII), thus were Triple Sugar Iron Agar, Simmon's citrate agar, Lysin decarboxylation broth, Indole test, Methylene Red, and Voges Proskauer test. This was carried out by taking the purified, colonies from the 24-h incubated nutrient agar plate and inoculating each tube (ISO 6579:2002(E), 2008).

Triple Sugar Iron agar

The tube of triple sugar iron agar was placed at an angle of 45^0 for slant and butt formation after sterilization of the media. The pure colony from nutrient agar was taken and stabbed at the center of the medium to the butt and strike on the slant of the TSI (HiMedia, India), then the tube was recapped loosely and incubated at 37^0 C for 24 hr.

After 24 hr. incubation, the positive *Salmonella* Colonies were produced red slant (alkaline), with yellow butt (acid) with blackening due to hydrogen-sulphide (H2S) production and sometimes gas production indicated by the formation of cracks and uplifting of the media from the bottom (Annex VII, A).

Simmon's Citrate Agar

Similarly, the tube of Simmon's citrate agar also placed an angle of 45^0 to prepare the slant and butt after sterilization. The pure colony from nutrient agar was taken and strike the surface of the slant of Simmon's citrate agar (HiMedia, M099, India) by zigzagging the straight loop back and forth. the tube was then recapped and incubated at 37^0C for at least 48 hr.

After incubation, *Salmonella* positive isolates show positive reaction which was indicated by the color change in media pH indicator bromothymol from green to deep blue on the slant (Annex VII, B).

Lysine Decarboxylase Broth

The sterilized lysine decarboxylate broth (HiMedia, M376, India) was inoculated with pure colony culture from nutrient agar and incubated at 37^0C for 24-48 hr. by maintaining anaerobic condition via adding 3ml of sterile paraffin oil.

The positive *Salmonella* isolate was shown a positive reaction in which the media gets back its normal purple color after anaerobic incubation (Annex VII, C).

Indole Test

After sterilization, 5 ml of the TSB (OXOID, CM0129, England) containing tube was inoculated with a pure colony from nutrient agar by using a sterile platinum loop and was incubated at 37^0c for 24 hours aerobically.

Then after 24 hours of incubation, 3 drops (0.5ml) of Kovac's reagent (Loba Chemie, LM0522A1503, India) was added and then the tube was gently shaken, kept for a minute, and was observed for any red color formation at the surface of the media. The *Salmonella* positive isolate was shown a negative reaction, in which there was no red ring formation at the surface of the media or yellow-brown ring was observed (Annex VII, F).

MR and VP Test

Both MR and VP tests are conducted on the same media called MR-VP broth or glucose phosphate peptone water. After sterilization, two tubes each containing 5ml of MR-VP broth (SRL, MM018, India) were inoculated with a pure culture of the test organism from nutrient agar. Then the tubes were incubated aerobically at 37^0C for 48hr.

After 48-h incubation, 3-5 drops of 1 % methyl red solution were added to the one of MR-VP broth containing tube for MR test. While the remaining one MR-VP tube was added by a very small amount of about 3 drops of alpha-naphthalol (Blulux, M.W.144.17, India). Subsequently followed by 3 drops of 40% KOH (Loba Chemie, 131058-3, India) and well shacked to mix for VP test (Annex VII, E).

The positive *Salmonella* colonies were showing immediate positive reaction while adding MR reagent for Methyl red test. This is indicated by red ring development on the top of the tube. Positive *Salmonella* isolates also showed a negative reaction for the Voges-Proskauer test that was indicated by no development of pink color (Annex VII, D).

Finally, all the biochemical confirmed pure *Salmonella* colonies were preserved at +4 until molecular detection process by using glycerol after the colonies grew on BHI (HiMedia, M211, India) agar slant at 37 ^{0}C for 24 hrs.

3.7.3. Molecular Detection and characterization

From the total of 88 biochemical test positive *Salmonella* isolates, 42 (~50%) representative samples from all farms were selected and subjected to further confirmation by molecular techniques at the National Veterinary Institute Molecular laboratory.

DNA extraction and purification

The preserved 42 (~50%) representative isolates were refreshed on tryptic soy broth and grown at 37°C for 24 hr. Then the bacterial culture was subjected to DNA extraction. The Genomic DNA of all *Salmonella* isolates was extracted from the culture using the DNeasy Mini kit (QIAGEN GmbH, 40724 Hilden, Germany) following the manufacturer's instructions (Annex IX).

Briefly, 200 µl of the bacterial suspension was added to the microcentrifuge tube followed by adding 200 µl lysis buffer (AL) and mixed by using pulse vortex for 10 sec. Then incubated for 10 min at 56 ^{0}C with 20 µl of proteinase K, followed by centrifugation, the addition of 200 µl of 97% ethanol, and vortexing. The lysate was then transferred carefully to the DNeasy Mini spin column contained in the collection tube and centrifuged at 8000 rpm for 1min. After centrifugation, the filtrates were discarded and the collection tube containing the spin column was changed by the clean one. Then subsequent washing steps and centrifugation steps were continued with the addition of 500 µl washing buffer solution-1 and centrifuge at 8000 rpm for 1min. Then the filtrates were discarded and replaced by the clean collection tube, followed by the addition of 500 µl washing buffer solution-2 and centrifuged at 13,400rpm for 3min. The spin-column was then placed on a clean Eppendorf tube and add 200 µl elution buffer was added, followed by 1 min incubation at room temperature and centrifugation at 8000 rpm for 1 min. Then the DNA was stored at −20°C until the amplification process.

PCR and Gel electrophoresis

Conventional PCR was performed for molecular identification of isolates on the extracted DNA by using pairs of serotype-specific primers targeting specific *Sdf*I gene as described by

Borges *et al.* (2017) with little bit modifications. The primer used to identify *S*. Enteritidis is presented in (Table 2).

The PCR amplification was carried out within PCR reaction tube of final volume 20 µl amplification mixture, consisting of 17 µl master mix that incorporates 3 µl of nuclease-free water, each of 2 µl of forward and reverse primers targeting *SdfI* genes, 10µl of ready-made iQ^{TM} Supermix (BIO-RAD, USA) and 3 µl of the prepared template DNA. Negative control was prepared by adding 3 µl of sterile nuclease-free water (Ambion AM9930, UK) and positive control was prepared by adding 3 µl of known positive.

Amplification was conducted in a thermocycler (BIO-RAD, C-1000 Touch™ Thermal Cycler Singapore) The cycle conditions consisted of an initial denaturation 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec, and elongation at 72°C for 60 sec, with 7 min final extension period at 72°C followed by holding 10°.

The amplified products were separated by running gel electrophoresis on TAE buffer (Fisher Scientific, US). The amplicons were first mixed with loading dye of 6x concentration (SIGMA, USA G7654) and 125x concentration intercalating dye or gel red (Biotium, Australia Cat:41003). Then the mixture was loaded in the pre-cast wells of 2% agarose (CSA-AG500, France) that submerged under buffer in the gel tank. A 1kbp (thermo-scientific, Lithuania) of DNA ladder was also used as a marker. The applied electrical current was 120 volts for 1:20 hr. The Fragments were then trans-illuminated with UV light (uvitec, serial no 08200913, UK) and imaged with a gel documentation system on uvitec software.

Table 2: Primers used for detection of *Salmonella* serotype

Bacteria	**Target gene**	**Primer sequence (5'-3')**	**Amplicon size**	**Reference**
S. Enteritidis	*SdfI*	F: TGTGTTTTATCTGATGCAAGAGG R: TGAACTACGTTCGTTCTTCTGG	304	(De Freitas *et al*. 2010)

3.7.4. Antimicrobial Susceptibility Testing

All the confirmed *Salmonella* serotype that was identified as *S.* Enteritidis on PCR were subjected to 10 different antimicrobials with known concentrations to determine the antimicrobial profile of *S.* Enteritidis (Table 3). The antimicrobials were selected based on their empirical use for the treatment of gram-negative bacterial infections and also lack of access to other antimicrobials. Antimicrobial susceptibility test was performed according to the National Committee for Clinical Laboratory Standards (CLSI, 2013). By using standard Kirby-Bauer disk diffusion method on Muller Hinton agar medium (Microxpress, AM5071, India). From each isolate, four to five well-isolated colonies grown on nutrient agar were transferred with the sterile loop into tubes containing 5 ml of Tryptone soya broth (OXOID, CM129, England). Then the broth culture was incubated at 35ºC for 4 hr. and adjusted to attain its turbidity by using sterile saline solution and by adding more isolated colonies until it achieved the 0.5 McFarland standards. The sterile cotton swab was dipped into the suspension and the bacteria were swabbed uniformly over the surface of the Muller Hinton agar plate.

Table 3: The break-point of Antibiotic disks used for Enterobacteriaceae with respective concentrations.

Antimicrobial agents	**Symbol**	**Disk content (µg)**	**Interpretive Categories and Zone Diameter Breakpoints (nearest whole mm)**		
			S	**I**	**R**
Ceftazidime	CAZ	30	≥21	18–20	≤17
Cefotaxime	CTX	30	≥ 26	23- 25	≤ 22
Chloramphenicol	C	30	≥18	13–17	≤12
Ampicillin	AMP	10	≥ 17	14- 16	≤ 13
Tetracycline	TE	30	≥ 15	12- 14	≤ 11
Nalidixic acid	NA	30	≥ 19	14- 18	≤ 13
Meropenem	MEM	10	≥ 23	20- 22	≤ 19
Sulfamethoxazole/Trimethoprim	SXT	25	≥16	11–15	≤10
Azithromycin	AZM	15	≥13	-	≤12
Tigecycline	TGC	15	≥19	15-18	≤14

S-sensitive, I- intermediate, R- resistant

Source: (CLSI, 2017)

The plates were held at room temperature for 3 min in a biosafety cabinet to allow drying. Then Antibiotic discs with the known concentration of antimicrobials such as Ampicillin (10µg), Cefotaxime (30µg), Chloramphenicol (30µg), Ceftazidime (30µg), Nalidixic acid (30µg), Sulfamethoxazole/Trimethoprim (25µg), Tigecycline (15µg), Meropenem (10 µg), Azithromycin (15µg) and Tetracycline (30µg) were placed by using sterile forceps and the plates were incubated for 24 h at 37ºC. The diameters of the zone of inhibition were measured with a caliper and recorded to the nearest millimeter (Annex VIII). Interpretation of the results (i.e. categorization of isolates into susceptible, intermediary, or resistant) was done according to NCCLS guidelines (Lacy *et al*., 2004).

3.8. Ethical Clearance

Ethical clearance was obtained from the animal research ethical review committee of Addis Ababa University, College of Veterinary Medicine (Certificate Ref. No: VM/ERC/09/04/13/2021) for collecting samples from poultry. Oral informed consent was also obtained from the animal owners. All the sampling processes from the animals were undertaken as per the standards approved by the animal welfare and research ethics committee at the College, which is under the international guidelines for animal care and welfare.

3.9. Data Management and Analysis

The obtained data were coded and entered into a Microsoft Excel sheet (Microsoft® Office Excel 2010) before being analyzed with R-Statistical software (version 3.6.2). The proportion of *Salmonella* isolates were calculated using descriptive statistics such as percentage and frequency distribution, in which the number of positives was divided by the total number of samples and multiplied by 100. The association between the occurrence of *Salmonella* with different risk factors was assessed by univariate and multivariate logistic regression analysis. The odds ratio (OR) was used to compare the strength of the association of the potential risk factors with the occurrence of the pathogen.

3.10. Constraints and Limitations of the Study

This study was initially proposed for molecular detection of *Salmonella* species followed by *Salmonella* serotypes detection and characterization. But due to different constraints like lack of species-specific primers as well as different other serotype-specific primers the above objectives were fulfilled partially. Additionally, the lack of commercial availability of different antimicrobials, and their high expenses, which descended with the emergency of COVID -19 pandemic leads to only a few antimicrobials to be used for antimicrobial sensitivity testing. Similarly, a very use full selective and differential media like Bismuth sulfate agar, for isolation of salmonella as well as different other confirmatory biochemical tests including different sugars test for differentiating purpose of the salmonella serotypes were also not included due to the above reasons.

4. RESULTS

4.1. Prevalence of *Salmonella*

In the present study, a total of 380 samples (260 cloacal swabs, 30 fecal droppings, 30 litter samples, 30 feed samples, and 30 water samples) were collected to isolate, confirm molecularly, and perform antibiogram profiling of *Salmonella* in and around Adama town. The overall prevalence of *Salmonella* in all the types of samples was 23.2 % (88/380) originated from broiler chickens and their environments. The occurrence of *Salmonella* in cloacal swab sample was 18.5% (48/260), pooled fecal droppings 50% (15/30), litter samples 43% (13/30), feed sample 16.7% (5/30), and water swab sample 23.3% (7/30) (Table 4).

The highest prevalence of *Salmonella* was recorded on the pooled fecal sample (50%) followed by litter (43%), water (23.3%), cloacal swab (18.5%), and feed (16.7%) samples. Statistical analysis of the data showed that there was a statistically significant difference ($P<0.05$) on fecal and litter samples with $p= 0.000$ and 0.001 respectively. However, there was no statistically significant difference ($P>0.05$) on the cloacal swab, water, and feed samples (Table 4).

Table 4: The prevalence of *Salmonella* in different sample types

Type of sample	Total no. of sample	No. of positive (%)	P-value
Cloacal swab	260	48(18.5%)	0.0601
Litter	30	13(43%)	0.001
Pooled Fecal dropping	30	15(50%)	0.000
Feed	30	5(16.7%)	0.936
Water	30	7(23.3%)	0.327
Total	**380**	**88(23.2%)**	

4.2. Univariate logistic regression

In the Univariate risk factor analysis, the odds of occurrence of *Salmonella* were 2.05 times significantly higher in Adama woreda (around Adama) chicken farms when compared to chickens which were found in the town. Comparing the types of samples, the odds of occurrence of *Salmonella* were 3.99 and 5.21 times significantly higher in litter and fecal samples, respectively as compared to the others. However, there was no statistically significant difference (P>0.05) between breeds on the occurrence of *Salmonella* (Table 5).

Table 5: Univariate risk factor analysis for the occurrence of *Salmonella* in poultry farms.

Variable	**Category**	**No of sample**	**No positive (%)**	**OR**	**95% CI**	**P-value**
Location	Peri-urban	195	57(29.2%)	2.05	1.26 -3.39	0.004
	Urban	185	31(16.7%)	-	-	-
Breed	Saso	210	43(20.5%)	0.72	0.44 - 1.15	0.169
	Cobb-500	170	45(26.5%)	-	-	-
Age	>2 months	90	27(30%)	1.61	0.94 – 2.72	0.07
	≤2 months	290	61(21%)	-	-	-
Sample type	Litter	30	13(43%)	3.99	1.75 - 8.98	0.001
	Fecal	30	15(50%)	5.21	2.32 - 11.78	0.000
	Feed	30	5(16.7%)	1.04	0.33 - 2.72	0.936
	Water	30	7(23.3%)	1.59	0.58 - 3.84	0.327
	Cloacal swab	260	48(18.5%)	-	-	-

OR= Odds ratio, CI= confidence interval

4.3. Multivariate logistic regression

The potential effects of different risk factors were also assessed using multivariate logistic regression in line with their degree of association using the Odds Ratio. Accordingly, the likely hood of isolation of *Salmonella* was 6.91 times significantly higher in the fecal sample

as compared to the other types of samples. Similarly, the likely hood of isolation of *Salmonella* in the chicken Sasso breed was 0.27 times significantly lower than that of Cobb-500. Moreover, the likelihood of occurrence of *Salmonella* isolation in chickens with >2 months of age was 2.58 times significantly higher than the chickens below 2 months of age (Table 6).

Table 6: Multivariate logistic regression analysis for the association between risk factors and *Salmonella* infection

Factors	Category	OR	95%CI	P value
Location	Urban	-	-	-
	Peri-urban	1.62	0.91 - 2.90	0.082
Sample type	Cloacal swab	-	-	-
	Litter	5.16	2.11 -12.72	0.00
	Fecal	6.91	2.85 -17.19	0.00
	Feed	1.24	0.38 -3.44	0.69
	Water	1.93	0.68 – 4.99	0.19
Breed	Cobb-500	-	-	-
	Sasso	0.27	0.12 -7.04	0.001
Age	≤ 2 months	-	-	-
	>2 months	2.58	1.26 -5.44	0.01

OR = Odds ratio, CI= Confidence interval

4.4. Molecular characterization by conventional PCR

Molecular characterization was done by convention PCR, in which the *SdfI* gene was amplified for the detection of isolated *Salmonella* Enteritidis. From the total of 42(~50%) representative samples being subjected to molecular characterization. Accordingly, 10(23.8%) samples were found to be positive to *S.* Enteritidis (Figure 4). From these, the highest detection rate was obtained in fecal dropping (42.85%) followed by cloacal swab (25%). Whereas both Water and Feed samples were negative to *S.* Enteritidis (Table 7).

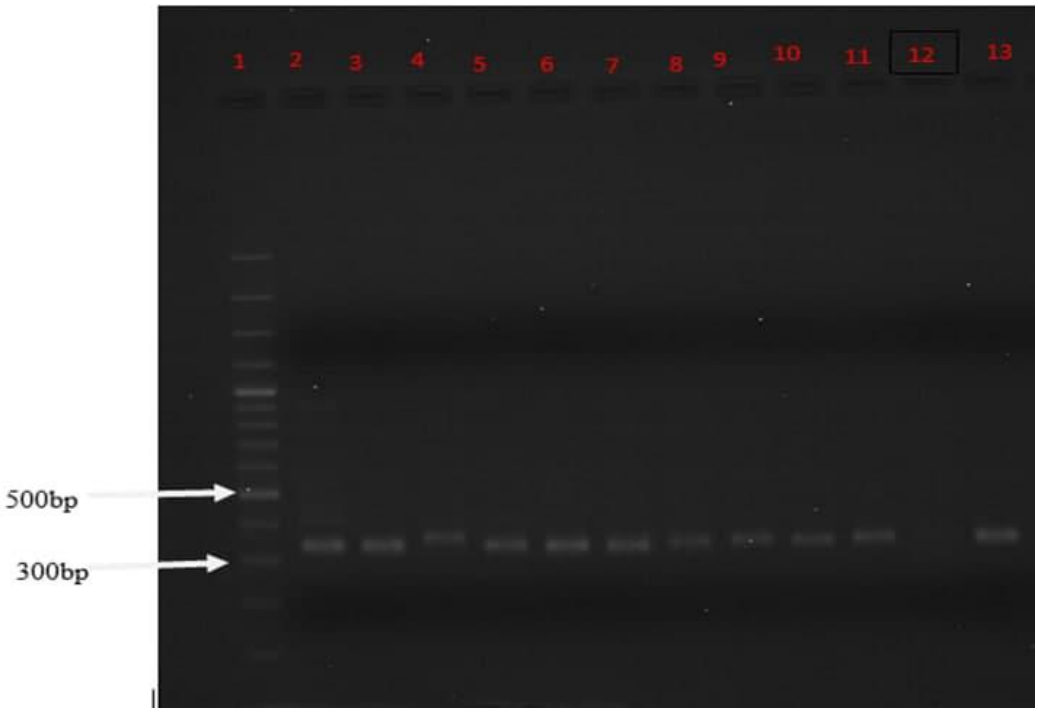

Figure 4: Conventional PCR for *Salmonella* Enteritidis

Lane 1 was 1 kb molecular ladder, Lane 2,3,4,5,6,7,8,9,10 and 11 were positive for *SdfI* gene that amplified at 304bp, Lane 12 and 13 were negative and positive control, respectively.

Table 7: Results of Conventional PCR positive samples per sample types

Sample type	**Number of samples tested**	**Conventional PCR positive (%)**
Cloacal swab	24	6(25%)
Litter	6	1(16.67%)
Fecal	7	3(42.85%)
Feed	2	0(0%)
Water	3	0(0%)
Total	42	10(23.8%)

4.5. In vitro antimicrobial susceptibility test result

The antimicrobial susceptibility test was performed on confirmed 10 *Salmonella* isolates (*Salmonella* Enteritidis) by using the disk diffusion method. The susceptibility degree ranges from 10% up to 100%. Similarly, resistance ranges from 0% up to 90% in tested *Salmonella* isolates. Out of 10 *Salmonella* isolates, all were resistant to the 8 antimicrobials tested except to Ceftazidime and Meropenem. The highest level of resistance was observed for Ampicillin (90%), Tetracycline (80%), and Nalidixic acid (70%) followed by Tigecycline (60%), Sulfamethoxazole/ Trimethoprim (40%), and Chloramphenicol (20%). Ceftazidime and Meropenem were the antimicrobials that show maximum sensitivity (100%) with no resistance profile to any of the tested isolates. Azithromycin and Cefotaxime also had maximum sensitivity (90%) followed by Chloramphenicol (80%) sensitivity (Table 8).

Table 8: Antimicrobial resistance profile of *Salmonella* Enteritidis

Antimicrobial used	**Disc concentration(µg)**	**Susceptibility and resistant pattern of 10 selected *Salmonella* isolates**		
		Resistant (%)	**Intermediate (%)**	**Susceptible (%)**
Tetracycline (TE)	30	8(80%)	0(0.0%)	2(20%)
Ceftazidime (CAZ)	30	0(0.0%)	0(0.0%)	10(100%)
Meropenem (MEM)	10	0(0.0%)	0(0.0%)	10(100%)
Tigecycline (TGC)	15	6(60%)	2(20%)	2(20%)
Sulfamethoxazole/ Trimethoprim (SXT)	25	4(40%)	0(0.0%)	6(60%)
Chloramphenicol (C)	30	2(20%)	0(0.0%)	8(80%)
Ampicillin (AMP)	10	9(90%)	0(0.0%)	1(10%)
Nalidixic acid (NA)	30	7(70%)	1(10%)	2(20%)
Azithromycin (AZM)	15	1(10%)	0(0.0%)	9(90%)
Cefotaxime (CTX)	30	1(10%)	0(0.0%)	9(90%)

From 10 *Salmonella* Enteritidis isolated, a multi antibiotic resistance pattern was observed in 8(80%) isolates. The highest number 37.5% (3/8) of isolates were resistant to four drugs in a combination of TE, TGC, SXT, and AMP; TE, TGC, AMP, NA and TE, TGC, NA, AZM, and five drugs with a combination of TE, TGC, SXT, AMP & NA; TE, TGC, SXT, C and AMP and TE, SXT, C, AMP, and CTX. Followed by 12.5% (1/8) isolates that were resistant to three drugs in a combination of TE, AMP, NA, and six drugs in a combination of TE, TGC, SXT, C, AMP, and NA in which the maximum MAR was registered (Table 9).

Table 9: Multi antimicrobial resistances (MAR) profile of the isolated *Salmonella* (Total no of *Salmonella* isolates=8)

Number of antimicrobials	**Antimicrobial resistance pattern (No.)**	**No. of isolates resistance (%)**
Three	TE, AMP & NA (1)	1 (12.5%)
Four	TE, TGC, SXT & AMP (1)	3 (37.5%)
	TE, TGC, AMP &NA (1)	
	TE, TGC, NA & AZM (1)	
Five	TE, TGC, SXT, AMP & NA (1)	3 (37.5%)
	TE, TGC, SXT, C & AMP (1)	
	TE, SXT, C, AMP & CTX (1)	
Six	TE, TGC, SXT, C, AMP & NA (1)	1 (12.5%)

5. DISCUSSION

The overall prevalence of *Salmonella* (23.2%) in different types of samples collected from broilers chicken farms found in and around Adama town in the current study was consistent with the previous studies reported by Boscán-Duque *et al.*, 2007 and Jano *et al.*, 2018 who reported 23% and 23.2% positive isolates of *Salmonella* from different samples in Venezuela and Benishangul Gumuz regional state, respectively. Similarly, the study conducted by Iwabuchi *et al*., 2011 in Japan and Khan *et al.*, 2018 in Trinidad, showed relatively consistent reports with current findings; reporting 20% and 20.5% of *Salmonella* positive isolates from chicken meat. Whereas the current result was much higher than the earlier findings of Aragaw *et al*., 2010 in Hawasa, Jaleta *et al*.,2016 in Mojo., Abdi *et al*., 2017 in Hawassa, Temesgen *et al*., 2020 and Dagnew *et al*., 2020 in Adama who reported 16.1%, 15.3%, 16.7%, 7.5%, and 2.9% of *Salmonella* prevalence, respectively by biochemical characterization. On the contrary, the higher prevalence of *Salmonella* was recorded by the previous reports of Alebachew and Mekonnen, 2013 in Jimma, Andoh *et al*., 2016 in Ghana, and Djeffal *et al*., 2018 in Algeria, reporting 42.7%, 47%, and 34.3% prevalence of *Salmonella* in chickens reared under a different management system. The disparity among indicated above reports with the current study is possibly associated with variation in techniques used to isolate *Salmonella*, sample type, sample size, age and breed of chicken, management system, hygiene status of the farms, and difference in geolocation (Tsegaye *et al*., 2016).

The present study revealed considerable variation of prevalence of *Salmonella* among different types of samples by biochemical characterization. Accordingly, the highest proportion (50%) of the *Salmonella* was significantly isolated from pooled fecal samples. Similarly, the occurrences of *Salmonella* were significantly increased by OR= 6.91 times in fecal dropping when compared to other types of samples. This finding was in agreement with different reports conducted by Jaleta *et al*.,2016 in Ethiopia, Kagambèga *et al.,* 2013 in Burkina Faso, and Ziyate *et al*., 2016 in Morocco who reported a higher prevalence of *Salmonella* isolates from fecal dropping. The highest recovery of the pathogen from fecal dropping in contrast with the other samples may since infected chicken become intestinal carriers and continuously excrete the bacteria with their feces. Shedding of *Salmonella*

through their feces for long periods results in environmental contamination (Borsoi *et al*., 2011).

The present study also revealed considerable variations in *Salmonella* isolates among the districts. The higher odds of occurrence of *Salmonella* in chicken farms found around Adama (peri-urban) when compared to chickens which were found in the town (urban). This variation could be due to the difference in the number of samples collected from the two sites, the difference in employed hygienic practices, the presence of well-trained farm personnel, and lack of awareness in rural areas may explain the difference in *Salmonella* occurrence among urban and peri-urban areas.

The result of the current study indicated that a higher proportion of samples collected from broiler chickens with the age of greater than 2 months were positive for *Salmonella*. This suggests that the possibility of getting *Salmonella* increases with age which might be dependent on environmental exposure. This result was in agreement with the previous reports from west Shewa, Ethiopia (Sorba *et al*., 2020).

The conventional PCR analysis in the present study revealed that 23.8% of *Salmonella* isolates were found to be *S.* Enteritidis. This finding was consistent with the previous reports of Asif *et al*., 2017 who identified 23.3% of *S.* Enteritidis by detecting *Spv*B gene from broilers chicken samples found in Pakistan, in eastern Turkey Arkali and Çetinkaya, 2020 also report 21.9% of *S.* Enteritidis detection rate by *Sdf*III gene from chickens sample. Whereas the current result was much greater than the earlier findings Adamu, 2017 who reported 7.1% of *S. Enteritidis* using the *SdfI gene.* The high isolation of *S*. Enteritidis may be since it is more invasive than other serotypes.

In the current study, from the total of 10 *S.* Enteritidis subjected to antimicrobial susceptibility test against ten antimicrobials. All the isolates (100%) were susceptible to Ceftazidime and meropenem. While 9 isolates (90 %) were susceptible to Cefotaxime and Azithromycin. This report coincides with a report of different studies from different countries which were conducted on isolated *Salmonella* from chickens and humans samples by Li *et al*., 2020 in China, Andoh *et al*., 2016 in Ghana, Jones *et al*., 2002 in India and Goossens *et al*., 1984 in Peru. The possible reason for the effectiveness of the above-listed third-

generation beta-lactams and new generation carbapenem antibiotics with high susceptibility or less resistance of isolates to those antimicrobials might be due to the recent introduction of the drug, rarely used or not widely used drugs in the developing country like Ethiopia in both veterinary and human medicine sectors (Wabeto *et al*., 2017).

In this study, a high level of resistance was seen on frequently used antimicrobials such as Ampicillin, Tetracycline, and Nalidixic acid. From the antimicrobials used in the current study, the highest percentage (90%) of *Salmonella* isolates were resistant to Ampicillin. This finding was in line with previous reports that recorded 96% and 100% ampicillin-resistant *Salmonella* isolates from the dairy farm in Batu and Addis Ababa, respectively (Abdulahi and Abunna, 2019; Addis *et al*., 2011). The high prevalence of resistant isolates of *Salmonella* for these antimicrobials was most likely due to their widespread use in the livestock and public health sectors. Their widespread use might be due to their less expensive cost and widely or easily accessible antimicrobials without prescriptions for the vulnerable groups of Ethiopia and other developing countries in contrast with the limited access and high expense of newer cephalosporins and quinolone medications. Furthermore, the propagation of such resistant isolates throughout a human being could result in serious problems or even death (Zewdu and Cornelius, 2009).

The present study also indicates that 8 out of 10 (80%) isolates were having multiple antibiotic resistances (showing resistance for more than two antimicrobials). This result has consistency with Abrar *et al*., 2020, Temesgen *et al*., 2020, and Addis *et al*., 2011 who report multi antibiotic resistance in *Salmonella* isolates from dairy farms and poultry as 80%, 86.6%, and 83.3% respectively. This development of MAR in poultry might be associated with the irrational use of antibiotics as feed additives or therapeutics during the production stage. Furthermore, in a developing country like Ethiopia farmers have the possibility to acquire antibiotics from a pharmacy without medical supervision (Adam *et al*., 2018).

From these resistance isolates, the most frequent resistance showed in four and five antimicrobials 3/8 (37.5%). While 1/8 (12.5%) of the isolates were resistant to three to six antimicrobials. The finding of *Salmonella* isolates resistant to six antimicrobials in the current study is highly supported by the finding from Ethiopia who reported MAR *Salmonella* isolates for six antibiotics from chickens (Sorba *et al*., 2020). Besides such uses

for treatment (therapeutic) and prevention (prophylactic uses), antimicrobials have been added in low dosages to animal feed to promote faster growth. Although more and more countries prohibit the use of antimicrobials as growth promoters, it remains common in many parts of the world and this leads to multiple antibiotic resistance. The high proportion of multi-antimicrobial resistance showed by the isolated *Salmonella* spp. maybe the result of the unjudicial use of different types of antimicrobial agents in poultry production with an aim to retard the bacterial infection. In addition, some antibiotics are being used unscrupulously in poultry feed and water by the feed millers and farmers, respectively, and these are also another cause for showing multidrug resistance. The findings of this study suggested that multidrug-resistant *Salmonella* spp. isolated from broiler farms might be an important concern for public health (Rahman *et al*., 2018).

6. CONCLUSION AND RECOMMENDATIONS

The overall findings of the current study pertaining to biochemical and molecular results revealed a higher prevalence of salmonellosis in the broiler chicken farms in the study areas justifying the presence of contamination in poultry farms. The findings based on the sample type examined revealed that the highest prevalence of *Salmonella* isolates was detected in fecal dropping in both biochemical and conventional PCR tests. The considerable number of *Salmonella* isolates obtained from the PCR analysis in the current study showed multi antibiotic resistance (MAR) for various antimicrobials that are commonly used in both veterinary medicine and public health sectors. The high recorded result of MAR *Salmonella* in this study suggests that the irrational use of antibiotics by farmers and preliminary chicken producers which leads to broiler chickens are the potential source for MAR *Salmonella* infection in humans.

Based on the above facts and concluding remarks, the following recommendations are forwarded:

- Further studies on molecular confirmation for the other serotypes and sequencing should be done to identify the gene that plays a role in resistance to particular antibiotics.
- There should be strict and rational drug use approaches by imposing restrictions on the use of antimicrobials that show recurring resistance in the study area, widely and easily accessible drugs. Above all utilization of drugs without prescription should be discouraged.
- It is highly advisable to apply continuous awareness creation and training for farmers and farm attendants about personal and farm hygiene, the risk of getting zoonotic diseases, and the risk of antimicrobial resistance.

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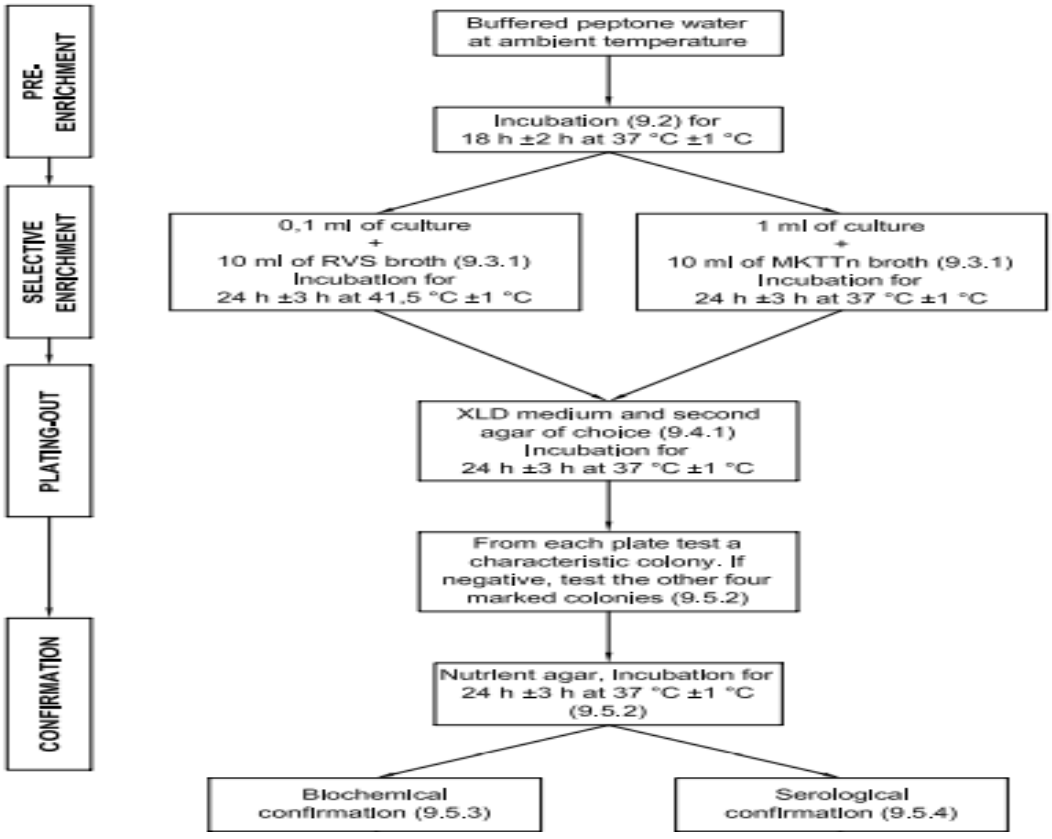
8. ANNEXES

Annex I: Sample collection shit

Farm code	Total chicken number	Total sample collected	age	breed	locality	Date of collection	Owner name	Telephone. no

Annex II: flow diagram



Source: ISO 6579:2002(E), (2008)

Annex III: Plating and biochemical result recording shit

Sample code	Date	Colony character On XLD	Biochemical test result								
			TSI				Simmon citrate	MR	VP	L-lysin	indole
			Butt	slant	gas	H_2S					

Annex IV: Types of media and reagents with their preparation used for isolation, biochemical test, and antimicrobial susceptibility test

Buffered Peptone Water Preparation

Preparation:

20 grams of BPW components were suspended in 1000ml of distilled water Mix well and distribute into a universal bottle of suitable capacity to obtain the portions necessary for the test and sterilize in an autoclave at 121 ℃ for 15 minutes. The final PH is 7.0± 0.2 at 25℃.

Composition (g/l):

- Peptone…….10.00
- NaCl……5.00
- Na2HPO4……3.50
- KH2PO4……...1.50

Rappaport Vassiliadis *Salmonella* Enrichment Broth

Preparation: 49.17 grams of hydrated medium RVS broth were suspended 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. Final PH 5.2 ± 0.2 at 25℃.

Composition (g/l):

- soya peptone …….4.5
- sodium chloride ……7.20
- potassium dihydrogen phosphate …….1.44
- magnesium chloride …….36
- malachite green …….0.036.

Xylose Lysine Deoxycholate Agar (XLD)

Preparation: Suspend 56.68 grams in one liter of distilled water. Heat with frequent agitation until the medium boils. DO NOT OVERHEAT. 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. Final PH: 7.4 ± 0.2 at 25 ℃.

Composition (g/l):

- yeast extracts …….3.0
- L-lysine hydrochloric acid …….5.0
- xylose …….3.5
- Sodium desoxycholate…….2.5
- 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

Nutrient Agar

Preparation: suspend 28 grams powder in 100ml distilled water. Then Heat, to boil, and 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 7.4 ± 0.2 at 25℃.

Composition (g/l):

- Agar…….15.0,
- Peptone……5.0,
- sodium chloride …….5.0,
- Yeast extract……2.0
- Beef extract…….1.0.

Triple sugar agar

Preparation: suspend 64.2 grams dehydrated medium in 1000ml of distilled water. Bring to boil to dissolve completely. Mix well and distribute into containers. Sterilize by autoclaving at 10lbs pressure or 115°C for 30 minutes. Allow the medium to set in sloped form with a butt about 2.5cm long. Final PH: 7.4 ± 0.2 at 25℃.

Composition (g/l):

- Beef extract …….3.0
- yeast extract ……3.0
- peptone ……20.0
- sodium chloride…… .5.0
- lactose …….10.0
- sucrose …….10.0
- Dextrose monohydrate …….1.0
- Sodium chloride ……5.000
- ferric sulphate ……0.2
- sodium thiosulfate …….0.3
- phenol red …….0.024

- agar …….12.0

Simmons Citrate Agar

Preparation: Suspend 24.28 grams in 1000 ml distilled water. Then Heat, to boiling, to dissolve the medium completely. Mix well and distribute in tubes or flasks. For tubes, dispense 4.0 to 5.0 ml into 16-mm tubes. Sterilize by autoclaving at 15 lbs. pressure (121°C) for 15 minutes. Cool in slanted position (long slant, shallow butt). Tubes should be stored in a refrigerator to ensure a shelf life of 6 to 8 weeks. The uninoculated medium will be deep forest green due to the pH of the sample and the bromothymol blue. Final pH (at 25°C) 6.8±0.2

Composition(g/L):

- Magnesium sulphate …….0.200
- Ammonium dihydrogen phosphate …….1.000
- Dipotassium phosphate ……1.000
- Sodium citrate …….....2.000
- Sodium chloride ……...5.000
- Bromthymol blue …….0.080
- Agar …….15.000

Lysine Decarboxylation broth

Preparation: suspend 14.02 grams powder in 1000 ml distilled water. Heat, if necessary to dissolve the medium completely. Dispense 5 ml amount into screw-capped test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool the tubed medium in an upright position and overlay with 2-3 ml of sterile mineral oil. Final pH 6.8±0.2 at 25°C

Composition (g/l):

- Peptone……5.000
- Yeast extract…….3.000
- Dextrose (Glucose)……...1.000

- L-Lysine hydrochloride…….…...5.000
- Bromocresol purple…….…....0.020

Tryptone soya broth

Preparation: Suspend 30 g to 1 litter of distilled water, mix well and distribute into final containers. Then Sterilize by autoclaving at 121°C for 15 minutes. Final pH 7.3 ± 0.2

Composition (g/l):

- Pancreatic digest of casein ……17.0
- Papaic digest of soybean meal ….....3.0
- Sodium chloride ……5.0
- Dibasic potassium phosphate ……2.5
- Glucose …….2.5

MR-VP Medium

Preparation: suspend 17.0 grams in 1000ml distilled water. Heat, if necessary, to dissolve the medium completely. Distribute in to test tubes 10ml amounts and sterilize by autoclaving at 15 Ibs pressure (121℃) for 15 minutes. Final pH 6.9 ± 0.2 at 25℃.

Composition (g/l):

- pancreatic digest of casein ……...3.50
- peptic digest of animal tissue …...3.50
- dextrose ……....5.00
- dipotassium phosphate …….5.00

Mueller Hinton Agar

Preparation: Suspend 38.00 g of the powder in 1000 mL distilled water. Heat, to dissolve. Then Sterilize by autoclaving at 121°C for 15 minutes. Final pH: 7.3 ± 0.1 at 25°C.

Composition (g/l):

- casein Acid Hydrolysate …….17.5

- Beef Extract Powder …….2.0
- Starch ……....1.5
- Agar …….17.0

Reagent preparation required for biochemical reaction

- **for Voges- Proskauer reaction**
    1. α-Naphthalol, ethanolic solution

Preparation: dissolve α-Naphthalol in ethanol

Composition (g/l):

- α-Naphthalol …….6 grams;
- ethanol 96 % (volume fraction) ……100ml.
    2. Potassium hydroxide solution

Preparation: dissolve potassium hydroxide in distilled water.

Composition (g/l):

- potassium hydroxide …….40g
- distilled water …….100ml

- **for antimicrobial susceptibility testing procedure where the bacterial suspension is compared to Standard McFarland, before swabbing on MHA media**
    1. 0.5 McFarland standards

Preparation: Add approximately 85Ml of 1% H2SO4 to a 100ml of the volumetric flask, using a 0.5ml pipette add 0.5ml of 1.17% BaCl.2H2O dropwise to the 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. Dispense three to seven ml, cub tube tightly and seal with paraffin and keep at dark and room temperature.

Composition:

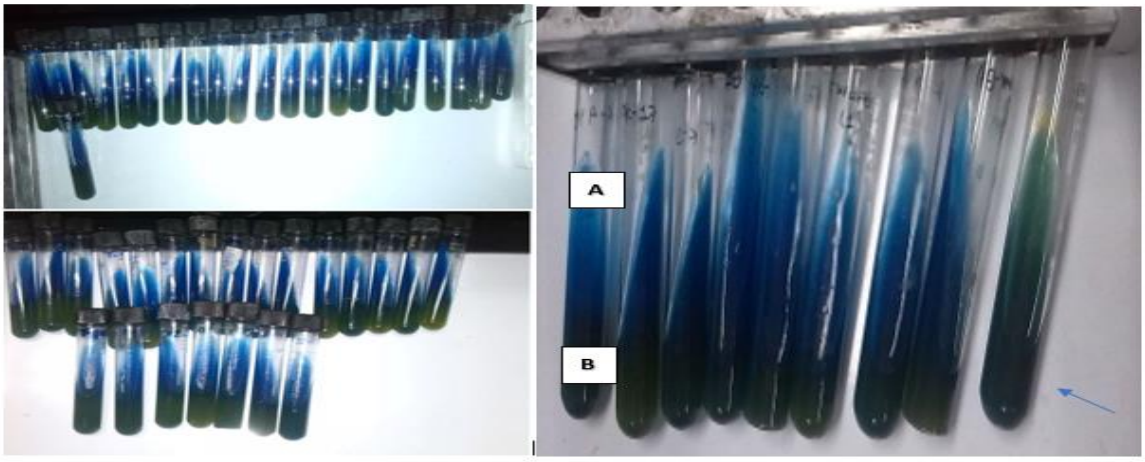
- 1.17% BaCl.
- $2H_2O$ solution and
- 0.36N of 1% sulfuric acid (H2SO4).

Annex V: Pictures were taken during sample collection

Annex VI: Pictures showing colony character of *Salmonella* on selective (XLD) media

Annex VII: Pictures showing biochemical results

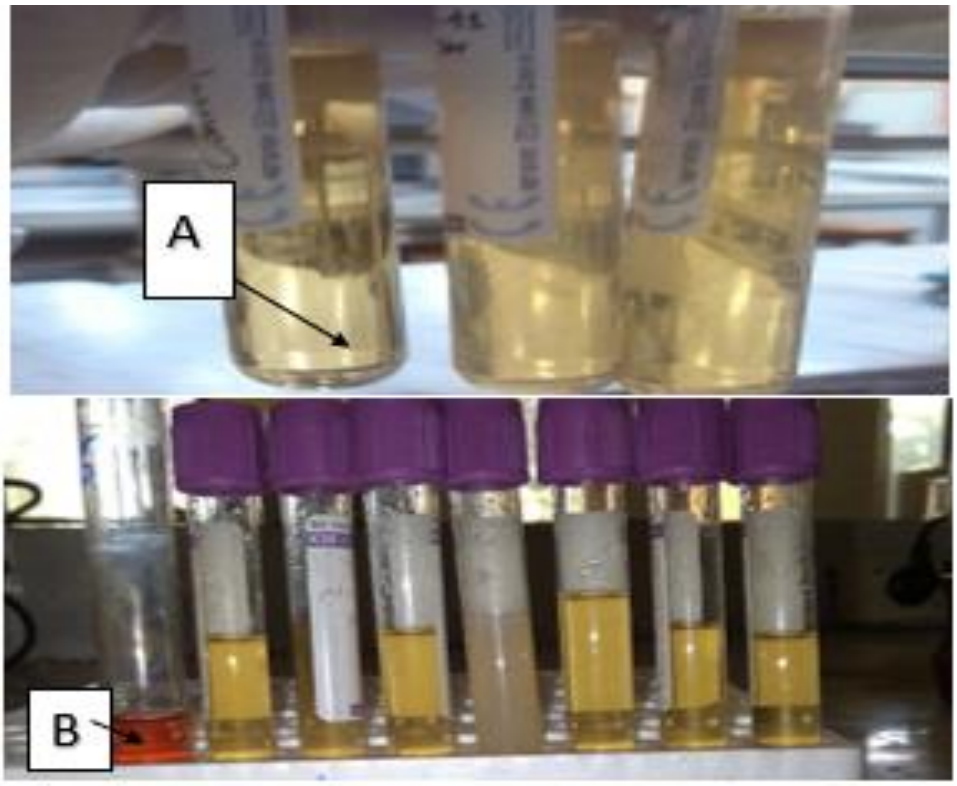
A. Triple sugar iron agar (TSI agar): Alkaline slant=A, Acidic butt=B, and Uninoculated (pointed by the arrow)



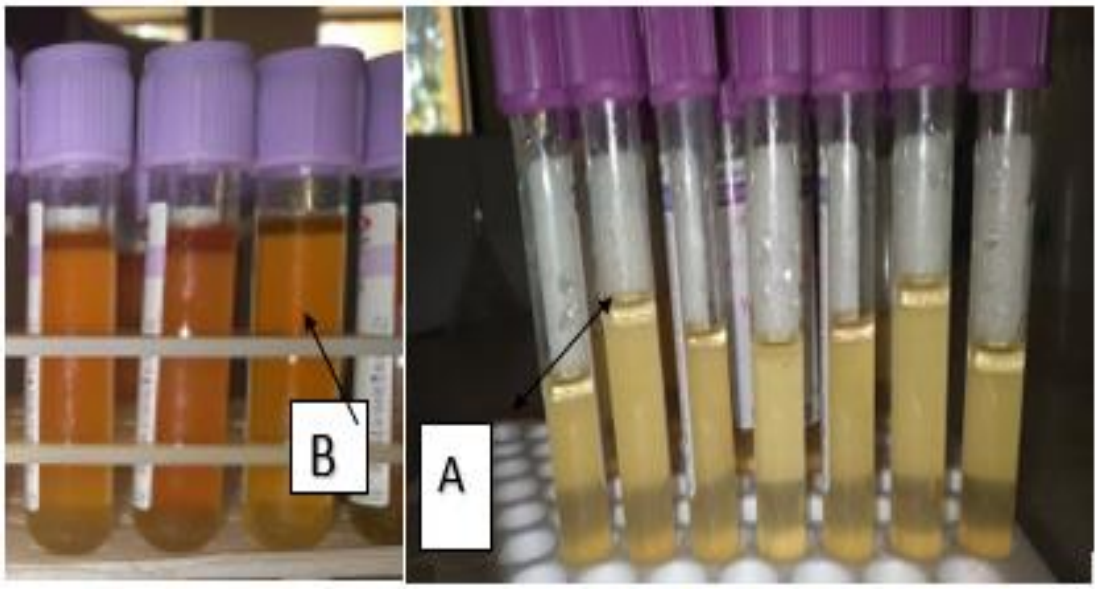
B. Simmon citrate agar: blue slant=A, green but=B and uninoculated(pointed by the arrow

C. L- -Lysine decarboxylation broth: uninoculated(negative control)=A, lysin decarboxylation negative sample=B and positive sample to lysin decarboxilation test= C

D. Methyline Red test: positive result= A and negative control(uninoculated)= B

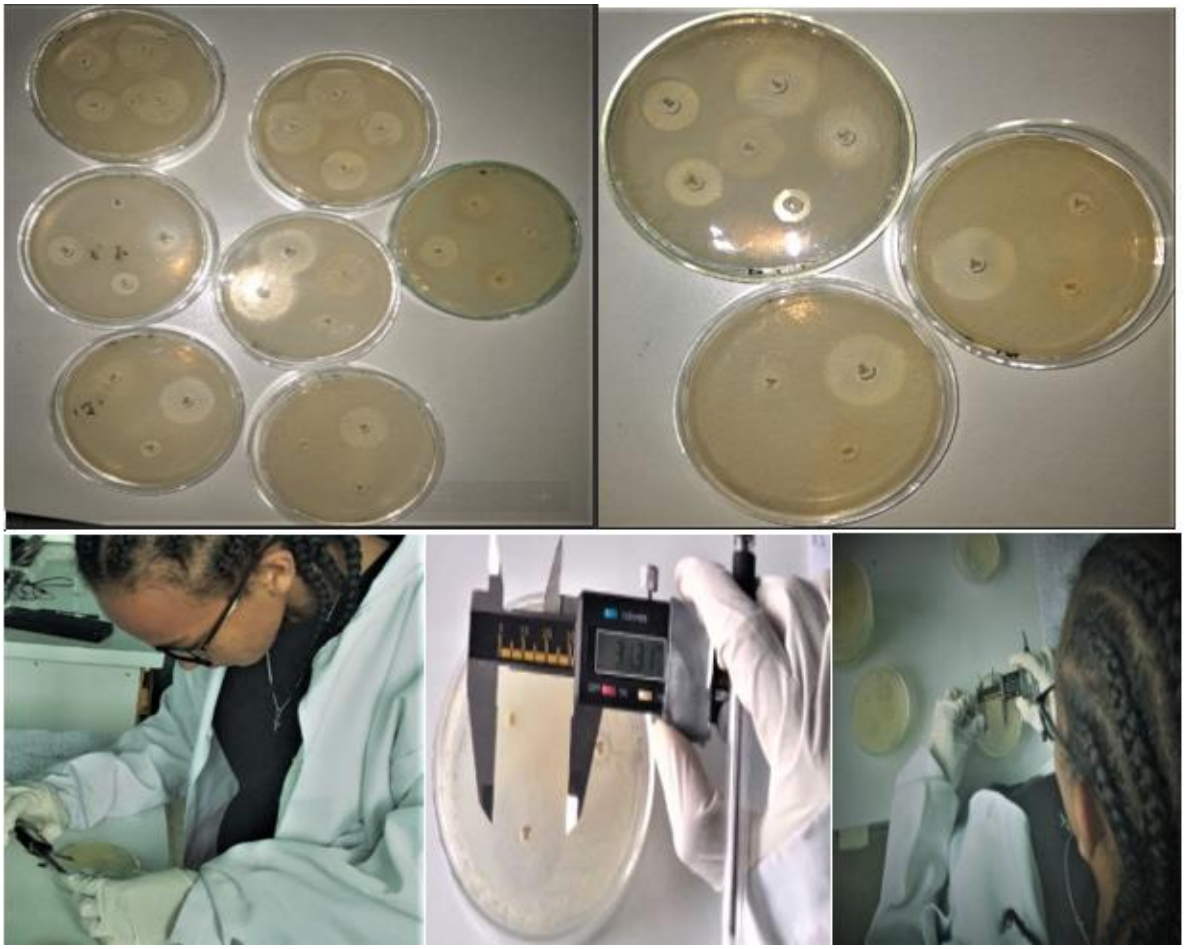


E. Voge-prausker test: negative control= A and positive control = B. while the rest samples are negative to VP test.



F. Indole test: indole negative result = A and indole positive control= B

Annex VIII: Pictures showing different degrees of the zone of inhibition during antimicrobial sensitivity tests



Annex IX: Extraction of Genomic DNA

1. Cultured cells: Centrifuge a maximum of 5 x 106 cells for 5 min at 300 x g (190 rpm). Resuspend in 200 µl PBS. Add 20 µl proteinase K. Proceed to step 2.

2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.

3. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.

4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.

5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.

6. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.

7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

8. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.

9. Optional: Repeat step 8 for increased DNA yield.

Annex X: PCR protocol

- Thaw all reagents on ice.
- Assemble reaction mix into 90 µL volume in thin-walled 0.2 mL PCR tubes.
- Add reagents in the following order: nuclease-free water, IQ Supermix, template, and primers.
- Gently mix by tapping the tube. Briefly centrifuge to settle tube contents.
- Prepare negative control reaction without template DNA.
- Prepare positive control reaction with the template of known size and appropriate primers.

***Salmonella* Enteritidis Master mix preparation**

Type of primer	The volume required for each rxn	Total volume for 42 rxn
SE-F	2 µl	84 µl
SE-R	2 µl	84 µl
Supermix	10 µl	420 µl
Nuclease free water	3 µl	126 µl
DNA Template	3 µl	
	22µl reaction volume for each sample	

PCR reaction steps:

- Initial Denaturation at 94°C for 5 min, per kb
- Denaturation at 94°C for 1 min,
- Primer Annealing at 55°C for 1 min,
- Extension at 72°C for 1 min.
- Final Extension at 72°C for 7 min.

35 cycles

- hold at 10 ^{0}C.

Then the PCR product was analyzed by using gel electrophoresis

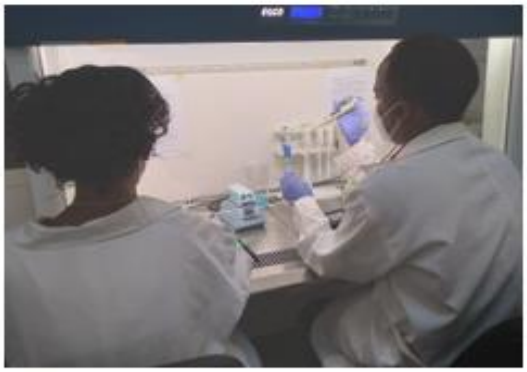
Annex XI: Agarose Gel electrophoresis

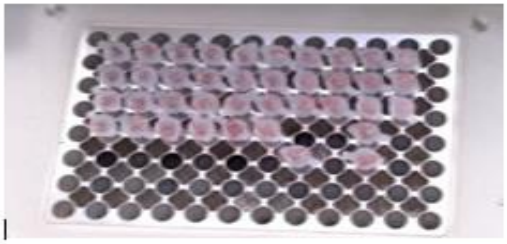
1. Measure 2 g of agarose in 100 mL of TAE buffer to make a 2% gel.
2. Mix agarose powder with 100 mL 1xTAE in a microwavable flask.
3. Microwave for 1-3 min until the agarose is completely dissolved
4. Let agarose solution cool down to about 50 °C for about 5 mins.
5. Pour the agarose into a gel tray with the well comb in place.
6. Place newly poured gel at 4 °C for 10-15 mins or let sit at room temperature for 20-30 mins, until it has completely solidified.
7. Add loading buffer to each of your DNA samples.
8. Once solidified, place the agarose gel into the gel box (electrophoresis unit).

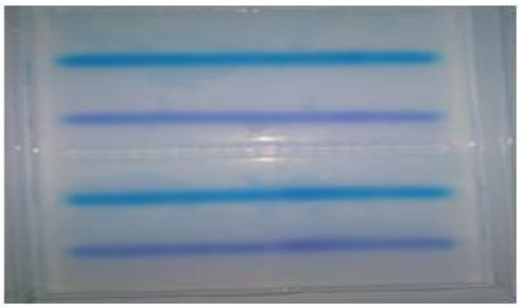
9. Fill the gel box with 1xTAE (or TBE) until the gel is covered.
10. Carefully load a molecular weight ladder into the first lane of the gel.
11. Carefully load your samples into the additional wells of the gel.
12. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.
13. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
14. Using any device that has UV light, visualize your DNA fragments. The fragments of DNA are usually referred to as ‘bands’ due to their appearance on the gel.
15. Using the DNA ladder in the first lane as a guide it is possible to infer the size of the DNA in your sample lanes.

Annex XII: List of photos captured during laboratory works







Annex XIII: Ethical Clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

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

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

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

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

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

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

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

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

Getachew Terefe (DVM, PhD) — Chairman

Signature

The Federal Democratic Republic of Ethiopia — Addis Ababa University College of Veterinary Medicine and Agriculture

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Please quote Our Ref. No. When Replying

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