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ISOLATION, ANTIMICROBIAL SENSITIVITY TEST, MALDI-TOF CONFIRMATION AND MOLECULAR CHARACTERIZATION OF *SALMONELLA* AND *ESCHERICHIA COLI* FROM COMMERCIAL POULTRY FARMS IN BISHOFTU, ETHIOPIA

A thesis submitted to College of Veterinary Medicine and Agriculture of Addis Ababa

University in the partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiology

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JUNE, 2023

BISHOFTU, ETHIOPIA

Approval sheet

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As MSc research advisor, I certify that I have read and evaluated the thesis prepared under my guidance by: Bilisuma Abebe: Entitled "Isolation, Antimicrobial Sensitivity Te st, MALDI-TOF Confirmation and Molecular Characterization of *Salmonella* and *Escher ichia coli* from Commercial Poultry Farms in Bishoftu, Ethiopia" and recommended that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: Bilisuma Abebe Entitled "Isolation, Antimicrobial Sensitivity Test, MALDI-TOF Confirmation and Molecular Characterizati on of *Salmonella* and *Escherichia coli* from Commercial Poultry Farms in Bishoftu, Ethiopia" and recommend that it is accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

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

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STATEMENT OF AUTHOR

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

Bilisuma Abebe

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Submission date: _________________

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
APEC	Avian Pathogenic *Escherichia coli*
AST	Antimicrobial sensitivity test
AW-1/AW-2	Washing agent
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
MALDI-TOF	Matrix-assisted Laser Desorption/Ionization Time of Flight
RVS	Rappaport Vassiliadis medium with Soya broth
PCR	Polymerase Chain Reaction
STEC	Shiga toxin producing *Escherichia coli*
SCV	*Salmonella* containing vacuole
SPI	Salmonella pathogenicity island
TSI	Triple Sugar Iron
TTSS	Typhimurium type secretion system

ABSTRACT

In Ethiopia, the poultry sector, which is becoming the main source of economic activity, is being challenged by infectious diseases and the frequent use of antibiotics which may results in antibiotic resistance that leads to economic crises and public health issues. The current research work was conducted from November 2022 to June 2023 with the objectives of identifying, antimicrobial sensitivity testing, MALDI-TOF confirmation and molecular characterization of pathogens from layer and broiler farms in Bishoftu, Ethiopia. A cross-sectional study design and rapid questionnaire were employed using random sampling method to collect a total of 284 samples (cloacal swabs, litter droppings, water, and feed) from diverse farm size. The rapid questionnaire survey was conducted to assess the commonly available antibiotics with regard to their usage practices and management in the selected commercial poultry farms. The samples were transported to laboratory and the isolates were identified using primary and secondary isolation and confirmed by MALDI-TOF and conventional Polymerase Chain Reaction (PCR). Out of 284 samples processed for both *Escherichia coli* and *Salmonella*, 40 (25 from layer and 15 from broiler) and 38 (12 from layer and 14 from broiler) samples were confirmed by primary isolation and biochemical tests respectively. From a total of 38 Confirmed isolates of *Salmonella* only 26 isolates were furtherly subjected to MALDI-TOF and PCR for confirmation and remained negative. Out of the 40 samples subjected to MALDI-TOF confirmation for *Escherichia coli,* 17 isolates were found to be positive. All confirmed isolates of *Escherichia coli* were further subjected to antimicrobial sensitivity test using nine types of antibiotics. The antimicrobial sensitivity test revealed highest resistance against tetracycline and ciprofloxacin in layers and amoxicillin in broilers. The findings of the present study disclosed that *Escherichia coli* to be widespread and prevalent in the study area with alarmingly high level of resistance for tetracycline where most of the farms were using it commonly. The rapid questionnaire on the use of antibiotics indicated indiscriminate utilization for prevention-treatment, for treatment and for treatment-prevention-growth purposes in a farm which might attribute to higher level of antimicrobial resistance. As a general, improvement of poultry biosecurity is recommended along with rational usage of antimicrobials. More specifically, the test agreement /discrepancy among the diagnostic tests should be further evaluated.

Key words*: Antimicrobial Resistance,* Biochemical tests, *Escherichia Coli, Isolation, MALDI TOF, Salmonella, Poultry*, PCR, Bishoftu

1. INTRODUCTION

Poultry production is the most important subsector of the livestock industry. It plays vital role in terms of creating job opportunities, providing nutrition helping as source of food, and as a means of investments especially for women (Shapiro *et al.,* 2015). Poultry farming is also a viable sector for poor family units because the business requires a small area of land and low investment costs for starting up and in succession of the operation. Furthermore, in Ethiopia, poultry are possibly a significant source of animal protein, contributing to nutrition and food security while also providing a source of income for huge part of the local community (Wubet *et al.,* 2019; Shapiro *et al.,* 2015).

Counting to the population of poultry in Ethiopia, there are about 54,495,026 total population of which 90.85% indigenous, 4.39% exotic and 4.76% hybrid breeds. There are various size and types of poultry flocks in Ethiopia and poultry sector contributes to the local economic development particularly, in per-urban of Ethiopia as more than half percent of families have poultry flock of different size. However, different mechanisms like genetic potential or low in indigenous breeds, high prevalence of infectious poultry disease and traditional way of feeding system affects the poultry production. Particularly, some of the pathogens affecting poultry production cause also food borne diseases contributing to public health (Abda *et al.,* 2021).

Countries all over the world are suffering from food borne disease outbreaks different bacteria type contributes to this in different ways (Abate and Assefa, 2021), and the emerging of antimicrobial resistant microorganisms is influencing the health world's capacity to treat bacterial-caused infectious disease effectively. Globally, 700,000 people die every year as a result of common bacterial antimicrobial resistance (Ibrahim *et al.,* 2019). This is thought to be the result of popular use of drugs for the last 50 years and then provide favorable conditions for resistance strains to survive (Muhie, 2019). 70% of antimicrobials produced globally are used in food animal production including poultry as growth promoter, protective and treatment of infection (Hedman *et al.,* 2020).

Infectious poultry diseases mainly caused by bacteria are the most common infections in attacking poultry and then lead to death, reduction in poultry production and significant economic loss and so accountable for a number of adverse social and economic effects The disease incidence is determined by numerous aspects including geographical climatic situation, poultry density, management situation, and status of immunization (AlMamun and Mehetazul 2019; Hossain *et al.,* 2021). Among many different types of disease-causing bacteria in poultry, *Escherichia coli* and *Salmonella* species are the most common ones (Phiri *et al.,* 2020).

Avian *Colibacillosis* and *Salmonellosis* are among the main infectious disease which can occur in all age ranges of birds (Mokgophi *et al.,* 2021). *Salmonella* is the most responsible bacteria in causing different types of acute and chronic diseases like pullorum disease, fowl typhoid and fowl paratyphoid. *Escherichia coli* is another common bacterium found in gastrointestinal tract of poultry as microflora, which means it is non-pathogenic and can be used as indicator for fecal contamination, but there are pathogenic strains of *Escherichia coli* (Martínez-álvarez *et al.,* 2022). Pathogenic strains of *Escherichia coli* cause a diversified lesion most commonly in immune compromised poultry. Cellulites, septicemia, Airscculitis, meningitis, endocarditic, yolk sac infection, swollen head syndrome, coli granuloma and *Colibacillosis* are the most common diseases caused by *Escherichia coli* (Hassan., 2014; Reza, 2009) and as a general both of them play a vital role in causing morbidity, mortality and economic loss in poultry industry of Ethiopia and worldwide (Mokgophi *et al.,* 2021).

Nowadays, an increase in resistance of *salmonella* to many antibiotics commonly used in poultry farms are becoming obvious in veterinary and public health centers in Ethiopia. This is thought to be the result of extensive use of drug in food source animals. Recently, reports on multi drug resistance of *salmonella* are becoming common (Dagnew *et al.,* 2020). Similarly, reports are there on a significant increase in resistance of *Escherichia coli.* High resistance profile reports on *Escherichia coli* are recorded for tetracycline, streptomycin, sulfamethoxazole, amoxicillin, nalidixic acid and ciprofloxacin (Abbassi, 2017). In the same manner reports on drug resistance status of *salmonella* shows resistance for ampicillin, chloramphenicol, nalidixic acid, tetracycline, trimethoprim/sulfa methoxazole, ciprofloxacin, and amoxicillin-clavulanic acid (Cabrera *et al*., 2004). On another report, *Salmonella* was found to be highly resistant to erythromycin, Spectinomy cin and streptomycin with showing low resistance to nalidixic acid, ciprofloxacin and norfloxacin (Mokgophi *et al.,* 2021).

In Ethiopia previously conducted studies also disclosed high level of drug resistance by *Salmonella* and *Escherichia coli* against the various drugs circulating in the country. However, the findings of the drug resistance manifested by *Salmonella* and *Escherichia coli* for a single antibiotic were quite inconsistent and different. Besides, the methodologi es used in the isolation of these pathogens were highly variable and dependent on conventional techniques. According to the study made by Abuna *et al,* 2016; Abdi *et al, 2017* and Abda *et al,* 2021 *Salmonella* is reported as resistant for ampicillin and chloramphenicol, but Dagnew *et al,* 2020 reported as susceptible for both antibiotics. In addition, Mohammed and Dubie, 2022 reported *salmonella* as susceptible for ampicillin and different isolate of *salmonella* with mixed resistance status. Martínez-álvarez *et al.,* 2022 reported *Escherichia. Coli* as resistant for gentamycin and chloramphenicol but Deressa *et al*., 2013 and Zeryehun and Bedada, 2013 reported as susceptible for both antibiotics. However, till the present no clear information was generated as to the antimicrobial resistance (AMR) status of both pathogens on the basis of production (broiler and layer commercial poultry farms) and breed type, molecular characteristics of AMR gene circulating in the study area. Even, there is no compiled and published data on the most commonly used antibacterial drugs in the study area. Given that the problem of AMR is a hot issue and unresolved problem still now, it requires continuous and intensive study. Studies that target the use of advanced diagnostic tools such as MALDI-TOF and PCR are essential to obtain reliable and valid data. In view of the above background information and existing research gaps, the present study was planned with the following objectives:

General Objective:

The general objective of the present study was to address the isolation, antimicrobial sensitivity test against the most commonly used antibiotics, MALDI-TOF and PCR confirmation tests of *Salmonella* and *Escherichia coli* from selected broiler and layer Commercial Poultry Farms in Bishoftu, Ethiopia.

Specific objectives:

1. To isolate *Salmonella* and *Escherichia coli* from various sample sources of selected broiler and layer Commercial Poultry Farms in Bishoftu.
2. To identify the most commonly used antibiotics and purpose of using them in both layer and broiler farms of Bishoftu.
3. To conduct antimicrobial sensitivity of *Salmonella* and *Escherichia coli* against different antibiotics and to compare resistance profiles of pathogens among the farms and sample type and
4. To perform confirmatory tests by using MALDI-TOF and PCR in selected broiler and layer Commercial Poultry Farms in Bishoftu, Ethiopia

2. LITERATURE REVIEW

2.1. Morphological Characteristics of *Salmonella* and *Escherichia coli*

2.1.1. Morphological Characteristics of Salmonella

The genus *Salmonella is a* group of rod-shaped, gram-negative, and facultative anaerobic, non-spore forming, motile, peritrichous flagella bacteria in the family Enterobacteriaceae (Salam *et al*., 2023) *Salmonell* is classified into two species namely, *Salmonella enterica* and *Salmonella bongori*, where *Salmonella enterica* contains the most clinically importa nt part of species. Classification of *Salmonella* as serovars and serotypes depends on the reaction of the bacteria to somatic (O) and flagella (H) antigens on the bacterial surface, and there are more than 2,579 serovars (Gut *et al*., 2018). These antigens are found on the bacterial cell membrane and are stable and able to form lipopolysaccharides and oligosac charides in the bacterial cell (Abdi *et al*., 2020). Except *for Salmonella gallinurum* and *Salmonella pullorum,* all serovars of the genus *Salmonella* are motile (Cosby *et al*., 2015). In addition, *Salmonella typhi* and a few other *Salmonella* serovars, including *Salmonella Dublin*, have a capsular polysaccharide virulence antigen (Heyndrickx. 2015). Various percentages of *Salmonella* isolates were found in Ethiopia, according to studies. Furthermore, a high proportion of *S. typhi* isolates are resistant to antimicrobial agents (Khadka *et al*., 2021). Based on differences in their 16S rRNA sequence analysis, the genus Salmonella is divided into two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is classified into six subspecies such as *arizonae, diarizonae*, *enterica*, *houtenae*, *indica,* and *salamae* (Popoff *et al.,* 2014).

2.1.2. Morphological Characteristics of Escherichia coli

Escherichia coli is a gram-negative, non-speculating, rod-shaped, facultative anaerobic and coliform bacterium pertaining to the genus *Escherichia* that commonly inhabits the environment, foods, and warm-blooded animals' lower gut (Basavaraju and Gunashree, 2022). *Escherichia coli* grow rapidly and duplicates in around 20 min under an optimum growth environment. It is used in gene manipulation system development because it can produce innumerable enzymes, and its fast-growing property helps to study the evolution of microorganisms. *Escherichia coli is* also found in the Enterobacteriaceae family and the *Escherichia* genus and has six well described pathotypes of the intestines as pathogenic strains based on their virulence properties and mechanisms of pathogenicity, including Shiga toxin producing *Escherichia coli* (STEC), enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC), enteroaggregative *Escherichia coli* (EAEC), diffusely adherent *Escherichia coli,* and enter invasive *Escherichia coli,* including Shigella strains (Jang *et al*., 2017). In addition, *Escherichia coli* can be classified on the basis of the serological identification of surface-associated antigens as O-somatic, K-capsular, and H-flagellar. Depending on this, there are at least 186 known somatic and 53 flagellar group antigens (Fratamico *et al*., 2016). Most of the time, they are considered common bacteria and microflora in the environment, foods, and intestines of humans and animals, and so are mostly harmless. But some strains are capable of acquiring resistance genes from the same or different species by horizontal gene transfer to become virulent and cause intestinal and extra intestinal diseases in both humans and animals (Kim *et al*., 2022).

2.2. Epidemiology of *Salmonellosis*

2.2.1. Geographical distribution of Salmonellosis

Salmonellosis is an avian infectious or clinical disease caused by *Salmonella* and plays an important role in the transmission of food borne disease to humans. Fowl typhoid and Pullorum disease are among the diseases caused by *salmonella,* and they are distributed worldwide and have economic significance. These are mainly founded in Latin America, the Middle East, and the Indian subcontinent, Africa, Southeast Asia, including Bangladesh, India, Pakistan, Nepal, and maybe other parts of the world. Fowl typhoid infects both commercial poultry and backyard chickens (Kabir, 2010). Another report indicates that pullorum disease has been eradicated from commercial poultry in developed countries such as Western Europe, the USA, Canada, and Australia but is still present in backyard flocks and wild birds. In developing countries, it is still among the major threatening diseases in poultry (Markos and Abdela, 2016). Antibiotic-resistant *salmonella* strains are prevalent in both developed and developing countries, and their emergence is encouraged mainly by the use of antibiotics as growth promoters in food animals and as treatments in veterinary medicine (Cui *et al*., 2021). *S.* Typhimurium serovar is the most prevalent and widely distributed serovar worldwide (Ferrari *et al*., 2019).

2.2.2. Host range in Salmonellosis

Pullorum disease affects specifically chicks under three weeks, and an excess number of chick deaths in the shell and immediate death after hatching can be its indication. Chickens, turkeys, quail, guinea fowl, pheasants, ducks, pigeons, sparrows, canaries, bullfinches, and parrots can be infected by *salmonella*, but pullorum disease is common mainly in chickens, turkeys, and pheasants (Markos and Abdela, 2016). *S. Gallinarum* and *S. pullorum* biovars are highly host-adapted, and chickens are used as their natural hosts. Fowl typhoid can be expressed as a per-acute, acute, or chronic disease and affects mainly adult chickens. Diseases, fowl typhoid caused by *S. Gallinarum* and pullorum disease caused by *S. pullorum* are among the most serious diseases in growing and adult chickens and poultry, and their vertical transmission complicates disease control (Berhanu and Fulasa, 2020).

2.2.3. Transmission of Salmonellosis

Transmission of *Salmonella* is very common and is persistent in dry conditions. Its transmission mechanism can be horizontal or vertical (Wibisono *et al*., 2020). Vertical transmission occurs when the reproductive organs of a chicken are infected by *Salmonella pullorum*. This can be caused by contamination of egg yolk, albumen, egg shell, and egg shell membranes before oviposition. On the other hand, the presence of pathogens in the ovules before ovulation is also another method of vertical transmission. As a general rule, *Salmonella* is able to colonize the lower part of the gut of chickens namely cloaca and persist in the spleen, ovary, and oviduct for a long period of time (Markos and Abdela, 2016). Chickens infected by the vertical transmission mode or by other vectors then transmit the disease by the horizontal transmission method. This can be through feed, water, or wild birds as carriers or vectors. Contaminated chickens can serve as carriers and shed the pathogen in feces, and the feces become the most important source of bacteria in the flock. Cannibalism between infected birds, mating, eating eggs, and skin wounds are other horizontal modes of transmission of *salmonella* in poultry (Berhanu and Fulasa, 2020). The susceptibility of hens to *Salmonellosis* and horizontal transmission in poultry can be increased by stress caused by feed and water shortages and inappropriate environmental temperatures (Gast *et al*., 2014).

2.3. Epidemiology of *Colibacillosis*

2.3.1. Geographical distribution of Colibacillosis

Colibacillosis is an acute respiratory and systemic infectious disease of poultry caused by avian pathogenic *Escherichia coli* (APEC) of subclass extra intestinal pathogenic *Escherichia coli* (ExPEC). This is a greater disease that causes economic significance to poultry farm owners worldwide (Guo *et al.,* 2020). The causative agent of *colibacillosis* is found all over the world and sometimes can be considered a normal microflora of a bird's gastrointestinal tract at a concentration of 106 per gram, and 10^5–10^6 per gram can be found in dust in poultry houses. All chickens in all age groups can be infected by *Escherichia coli*, but a high prevalence was reported in adult layers in Gazipur, Bangladesh, and a higher prevalence in broilers was reported in the large Mymensingh district of Bangladesh, the state of Kassala, and East Sudan (Panth, 2019).

2.3.2. Host range of Colibacillosis

Colibacillosis is reported as having severe economic significance around the world and is mainly affecting chicken layers. It can also cause osteomyelitis in turkeys (Dziva and Stevens, 2008). In humans, it is characterized by colonizing the gastrointestinal tract, especially in infants immediately after birth (Kaper *et al*., 2004).

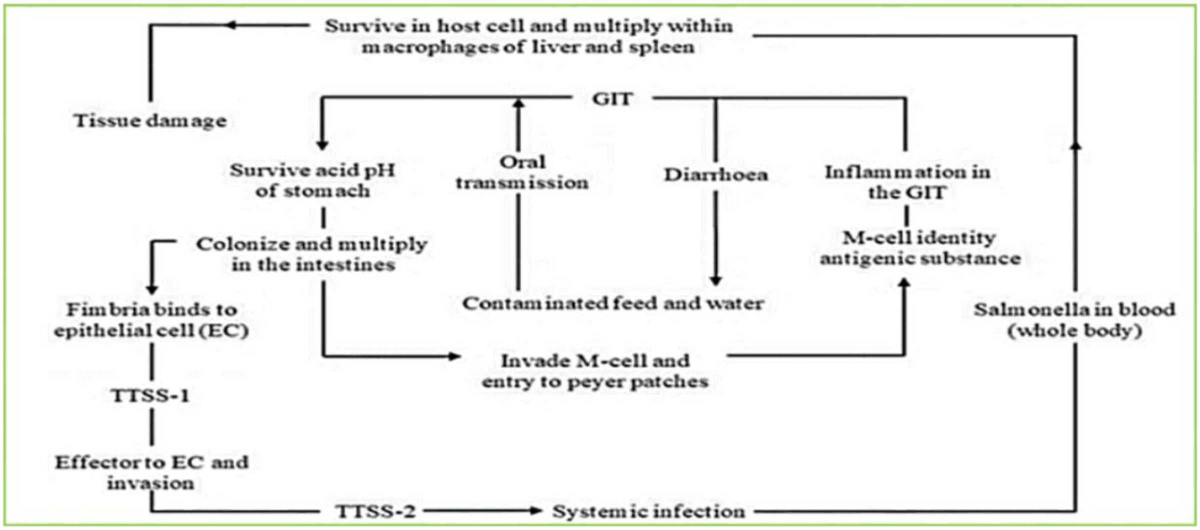
2.3.3. Transmission in Colibacillosis

As *Escherichia coli* is the normal microflora in the intestinal tract of poultry, it can be disseminated in the bird's environment by droppings and infect other birds by the oral fecal route of transmission. Therefore, birds' intestines and their environment are the most common reservoirs for strains of *Escherichia coli* that are able to cause infection. Flies, insects, mites, rats, and wild birds play an important role in transmitting *Escherichia coli,* and its transmission can be horizontally (directly or indirectly) or vertically (Olakolu *et al*., 2020). Vertical transmission is possible by transferring the pathogen from the carrier breeder to their progeny through the ovary or other reproductive organs (Panth, 2019).

2.4. Pathogenesis of *Salmonella* and *Escherichia coli*

2.4.1. Pathogenesis of Salmonella

S. Gallinarum, *S. pullorum,* and *S. enteritidis* colonize chickens with the help of virulence plasmids, which are localized on chromosomes and named pathogenicity islands (IIyas *et al*., 2017). During the occurrence of early infection, SPI-1 initiates the type 3 secretion systems, which then translocate effectors cells to help the bacteria invade the epithelial cells of the host intestine and initiate inflammation (Lorkowiski *et al*., 2014). When effectors are introduced into the host epithelial cell, *salmonella* changes the cell function of its host for the purposes of survival, replication, and transmission (Fuentes *et al*., 2008). When the systematic infection is there, *salmonella* uses the *Salmonella*-containing vacuole, which is a membrane-bound vesicle used in the process of internalizing *Salmonella* pathogenicity island-2. In this case, *Salmonella* dispersed in all fluids found in the body and organs like the liver, spleen, bone marrow, and other organs with high phagocytic cells (Barrow *et al*., 2012; Foley *et al.,* 2013).



Source: (Mkangara *et al*., 2020)

Figure 1: Schematic representation for Salmonella infection

Main steps in *Salmonella* pathogenesis are: contaminated water and feed are orally transmitted →gastrointestinal inflammation→diarrhea and shedding of *salmonella* to environment; *Salmonella* colonize and multiply in gut epithelial cells→expression of TTSS-1→effectors are injected and *Salmonella* is internalized into deeper tissue→expression of TTSS-2→systemic infection→multiplication of *Salmonella* in macrophages of reticuloendothelial system (Mkangara *et al*., 2020).

2.4.2. Pathogenesis of Escherichia coli

Pathogenic *Escherichia coli cause* diseases by invading tissues and causing toxigenesis. Adhesion, initial proliferation, and extracellular production of substances used to enable invasion are used in colonizing mechanisms that help to bypass the defense mechanisms of their host. Receptor and ligand on the cell surface of the host involved in the adhesion of bacteria. Molecular structures on the cell surface of a bacteria involved in pathogenicity are the capsule, glycocalyx, S layers, peptidoglycans, lipopolysaccharides, flagella, pili, and fimbriae (Manu *et al*., 2011). The infection caused by *Escherichia coli* is displayed as septicemia based on the virulence status of the strains involved in the infection, host status, and presence and type of predisposing factors, which then lead to sudden death and/or local inflammation in many organs. Perihepatitis, Airscculitis, and pericarditis are among the most common lesion types (Dziva and Stevens, 2008).

EPEC was the first well-described pathotypes of *Escherichia coli* and was known to cause infant diarrhea. Its pathogenesis depends on the principle called ‘attaching and effacing'. It attaches to the epithelial cells of the intestine intimately and causes striking cytoskeletal changes; it accumulates polymerized actins; intestinal microvilli will be effaced; and a pedestal-like structure will be raised from the epithelial cells. The ability to induce "attaching and effacing’ is facilitated by 35 kb. Pathogenicity Island, which is called the locus of enterocyte effacement (Kaper *et al*., 2004).

2.5. Public health importance of *Salmonella* and *Escherichia coli*

Salmonella genus represents the most common food borne pathogens frequently isolated from food-producing animals that are responsible for zoonotic infections in humans and animal species including birds. Thus, *Salmonella* infections represent a major concern to public health, animals, and food industry worldwide. In *Salmonella* infection in poultry which is caused by serovar enteritidis with a particular preference for the chicken reproductive system, vertical transmission is of great concern. In this case it happens by transovarian infection when the mother bird has a systemic infection that results in ovary infection and egg production in the oviduct. Bacteria migrating from cloaca into reproductive organs contribute to serovar enteritidis gaining access to eggs (Anamaria et *al.,* 2018).

Escherichia coli are bacteria that are commonly found in the lower intestine of warm-blooded organisms. Most *Escherichia coli* strains are harmless, but some can cause serious food poisoning. Shiga toxin-producing *Escherichia coli* (STEC) is a bacterium that can cause severe food borne disease. There are more than 700 serotypes, and the most common pathogen is O157:H7. The most common non-O157:H7 serotypes associated with disease from animals to humans include O26:H11, O103:H2, O111: NM, and O113:H21 (Nataro and Kaper, 2021).

2.6. Laboratory diagnosis of *Salmonella* and *Escherichia coli*

2.6.1. Laboratory diagnosis of Salmonella

The isolation of *Salmonella enterica* from ordinarily sterile clinical samples, often blood and bone marrow, is required for the conclusive diagnosis of enteric fever. The diagnosis is confirmed by culture, which also offers an isolate for molecular characterization, epidemiologic type, and testing of an antibiotic's susceptibility. Blood cultures are positive in 80% or more of untreated enteric fever patients. The yield from blood culture can be as low as 40% in endemic locations where antimicrobials are commonly taken prior to examination. In these circumstances, bone marrow aspirate culture is typically thought of as the reference standard approach, with a sensitivity of >80% (Watson, 2018).

The quantity of blood drawn for a blood culture is crucial since it affects how many germs are present there. Every milliliter of blood contains less than 10 live germs, and typically only one or less. Although cultures can still be positive in the third week in the absence of antibiotic exposure, the first or second week of the illness is thought to be the best time for detecting organisms circulating in the bloodstream (Escamilla e*t al.,* 2021). Studies on quantitative bacteriology have demonstrated that counts decrease when a sickness lasts longer (Simanjuntak, *et al.,* 2018).

In the sera of people with suspected enteric fever, the Widal test evaluates agglutinating antibodies against the LPS (O) and flagellar (H) antigens of Salmonella serovar Typhi. Although typically discouraged due to accuracy, it is nonetheless commonly utilized in some countries and is easy and affordable to do (Mantur *et al*., 2020). Lack of reagent uniformity and incorrect result interpretation has made the approach less effective. The typical antibody response to Lipopolysaccharides, flagella, vi capsular polysaccharide, or outer membrane protein antigens during enteric fever has been investigated using ELISAs (Simanjuntak, *et al.,* 2018). For the identification of Salmonella serovars Typhi and Paratyphoid A, primarily in blood, nucleic acid amplification assays, such as traditional PCR and real-time PCR, have been developed (Edelman and Levine, 2022). Methods for nucleic acid amplification have the capacity to amplify single organisms, nonculturable microorganisms, and dead organisms. Testing for bacterial antigens such Vi, O9, and Hd in urine has been used to diagnose enteric fever, however the results have been inconsistent. The VI antigen had the highest level of sensitivity, whereas the O9 and Hd antigens had lower levels (Edelman and Levine, 2022).

When additional samples were tested, the sensitivity for VI rose, but the specificity was l ess than satisfactory, especially in individuals with brucellosis. When between one and th ree urine samples were evaluated, the sensitivity in investigations of patients from Indone sia on the identification of O9 antigen in urine using an ELISA and dot blot format increa sed from 65% to 95% (Simanjuntak, *et al.,* 2018).

2.6.2. Laboratory diagnosis of Escherichia coli

Bacterial culture one of the methods used for the isolation of live organism in which samples like feces and intestinal content are used. The advantage of this method is the bacteria are easy to grow in one day, easy to do in any lab including in-house and is relatively low cost. However, some *Escherichia coli* are part of normal intestinal flora and those animals previously treated with antibiotics can prevent bacterial growth (Dziva and Stevens, 2008). Antimicrobial susceptibility test is another method used for diagnostic purpose which is test's in vitro ability of live organism to grow under specific concentrations of different antimicrobials. Sample such as feces and intestinal content are used and susceptible pathogens are possible good choice for treatment if antimicrobial can reach target tissue whereas, resistant selects different antimicrobial (Zhang *et al*., 2022). Biochemical methods are also the most appropriate biochemical tests conducted include Indole test, Methyl Red-Voges Proskauer (MR-VP) test, and Citrate Utilization Test. The tests were conducted by taking a pure colony from nutrient agar. The Indole test determines the ability of bacteria to split tryptophan molecules and produce Indole as an end product of the metabolic degradation of tryptophan amino acids, which has a positive reaction for *Escherichia coli* (Geletu *et al*., 2022; Naser, 2016).

Histopathology can also be used to evaluate the presence of tissue lesions or damage which can confirm the presence of disease. Sometimes it can also detect the presence of organisms directly bacteria through additional special staining. Tissue sample used the appropriate sample in which positive tests show strong association with accusation if able to demonstrate intestinal lesions with *E. coli* adhered to epithelial cells. Genotyping is a PCR technique that detects presence of specific sequence of DNA associated with known virulence genes. Genotyping is very valuable in identifying if isolated *E. coli* contains any of the virulence factors that would support the isolate as pathogenic (Batista *et al*., 2016). MALDI TOF can also be conducted for more confirmation of *Escherichia coli* and *Salmonella* as protocol recommended by Zhang *et al*., 2022 and Bruker MALDI Biotyper Manufacturer.

2.7. Drug Resistance Profile of *Salmonella* in Ethiopia

Tadesse *et al*. (2019) report multidrug resistance up to 72.72% and susceptibility of *Salmonella* isolates from laying hens and eggs to Gentamycin, Kanamycin, and Streptomycin. Research done in Modjo, central Oromia, reports a multidrug-resistant *Salmonella* isolate with 94.73%; another isolate from the same report is resistant to tetracycline; some other isolates are resistant to both tetracycline and Kanamycin; and again, other isolates are susceptible to Ciprofloxacin and Gentamycin (Abuna *et al*., 2016). Another report from the poultry industry in Southern Ethiopia shows that there are 100% resistance *Salmonella* isolates to Kanamycin and sulfamethoxazole-trimethoprim and different resistance levels to nalidixic acid, ampicillin, cefoxitin, streptomycin, tetracycline, chloramphenicol, and ciprofloxacin, and 0% resistance level for Gentamycin with 93.4% multi-drug-resistant isolates Abdi *et al*., (2017). Dagnew *et al*., (2020); Mohammed and Dubie, 2022; Belachew *et al*., (2021); and Abda et al., 2021 do have the same report on *Salmonella* as resistant to tetracycline and Kanamycin and susceptible to gentamycin.

Table 1: Drug Resistance Profile of Salmonella in Ethiopia.

Tetracycline *(30µg)*	Ampicillin (10µg),	Cefoxitin (30µg)	Ceftriaxone(30µg)	Chloramphenicol	Ciprofloxacin (5µg),	Nitrofurantoin (100	Gentamycin (10µg),	Kanamycin (30µg),	Nalidixic acid (30µg)	Neomycin (30µg)	Streptomycin (10µg)	Sulphathiazole trimetho prim 1.25/23.75 µg	Study area	Production type	Breed type	Reference
R	R	R	S	R	I	R	S	R	R	R	R	R	Modjo	-	-	(Abunna et al., 2016)
R	S	-	-	S	S	R	S	R	S	R	R	S	Adam a Mod	-	-	(Dagnew et al., 2020)
R	R	R	-	R	R	-	S	R	R	-	R	R	Southern Ethiopia	-	-	(Abdi et al., 2017)
R	S	M	-	M	S	-	S	R	M	-	R	M	Addis Ababa	-	-	(Mohammed & Dubie, 2022)
R	M	-	-	R	-	-	S	R	R	-	M	R	Central Ethiopia	Broiler	-	(Belachew *et al.*, 2021)
-	R	-	S	R	R	-	S	R	R	-	R	R	Kefa Eth iopia	-	-	(Abda*et al.*, 2021)

Key: (R) Resistant, (S) susceptible, (I) intermediate (M) mixed susceptibility resistant with intermediate, (-) not applicable or not clearly mentioned in the article.

2.8. Drug Resistance Profile of *Escherichia coli* in Ethiopia

A Meta-analysis report on drug resistance status of *Escherichia coli* in Ethiopia shows a high resistance rate of *Escherichia coli* at 83.81% for ampicillin, 75.79% for amoxicillin, 67.18% for tetracycline, 57.47% for trimethoprim-sulfamethoxazole, 56.69% for cephalothin, and 13.55% for Nitrofurantoin, which is relatively low resistance (Tuem *et al*., 2018). Similarly, Zeryehun and Bedada (2013) report high resistance of *Escherichia coli* to tetracycline, ampicillin, and amoxicillin, but no resistance was observed for gentamycin. Sarba *et al*., (2019); Martínez-álvarez *et al*., (2022); Bushen *et al*., (2021); and Deressa *et al*., (2013) commonly reported *Salmonella* as resistant to tetracycline. On the other hand, Sarba *et al*., (02019; Martínez-álvarez *et al*., (2022); and Bushen *et al*., (2021) do have a common report on *Salmonella* as resistant to chloramphenicol and sulfathiazole-trimethoprim. Conversely, Deressa *et al*. (2013) and Zeryehun and Bedada (2013) reported *Salmonella* as susceptible to chloramphenicol.

Table 2: Current Drug Resistance Profile of *Escherichia coli* in Ethiopia.

Tetracycline (30µg)	Norfloxacin (10	Chloramphenicol (	Ciprofloxacin	Nitrofurantoin	Gentamycin	Kanamycin (30µg),	Streptomycin	Sulphathiazole trim ethoprim 1.25/23.75 µg	Amoxicillin (20	Cefuroxime/ceftazi dim30µg)	Study area	Production type	Breed type	Reference
R	R	R	R	R	R	R	R	R	R	R	Amb o	-	-	(Sarba *et al.,* 2019)
R	-	R	R		R	-	-	R	-	R	Gondar	broiler	-	(Martínez-álvarez *et al.*, 2022)
R	-	R	-	-	-	-	-	R	R	-	Southwes t Ethiopia	-	Chicken	(Bushen*et al.*, 2021)
R	-	S	-	-	S	-	R	-	-	-	Kombolcha	-	White leghorn, rhodeIsland	(Deressa *et al.,* 2013)
R	-	S	R	-	S	-	R	-	R	-	Eastern Hararg	Broiler	-	(Zeryehun & Bedada, 2013)

Keys: (R) resistance, (S) susceptible, (-) not applicable or not mentioned in the article.

2.9. Impacts of Drug Resistance

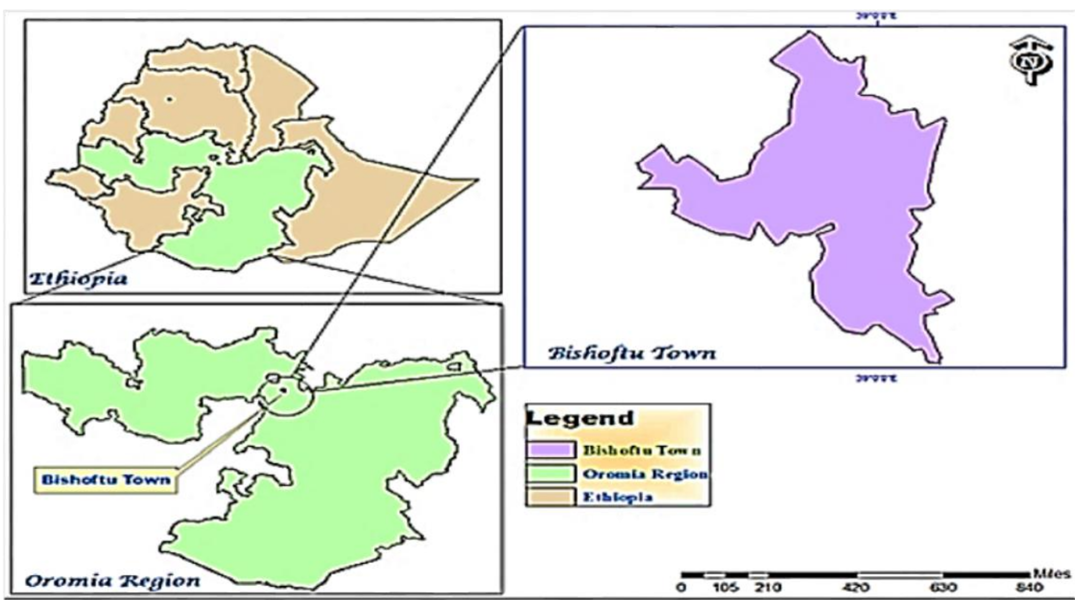
Bacterial mutation causes antimicrobial-resistant strains, and inappropriate use of antibiotics in this situation leads to failure in treatment and, in turn, increases morbidity, mortality, premature death, economic loss, financial burden on developing countries (Nhung *et al*., 2017; Ibrahim *et al*., 2019), and loss and/or loss in animal production (Spicknall *et al*., 2013). The quick and continuing spread of antibiotic-resistant microorganisms challenges the ability to successfully treat an increasing number of bacterial infectious diseases (Muhie, 2019).

Antibiotic resistance is associated with high medical costs and a significant influence on antimicrobial effectively. This aggravates the obstacle of disease control by creating possibilities for the distribution of resistant pathogens, decreasing treatment efficacy, and resulting in long-term infection. Being resistant to commercially available drugs forces one to use expensive therapy. The effectiveness of antibiotics is affected by resistance status differences among pathogens and public hygiene quality. Development of global trade and tourism helps in the distribution of multidrug resistance globally and has impacts on the import and export of different types of products, leading to a decrease in the economies of developing countries (Tanwar *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Study Area Description

The current research was conducted from November 2022 to June 2023 in Bishoftu town in selected layer and broiler commercial poultry farms. Bishoftu is located at 47 kms south-east of Addis Ababa. The city is positioned at 90^0 N latitude and 40^0 E longitudes at an altitude of 1550 meters above sea level in the middle highlands of the country. The city has a yearly rainfall of 892 millimeters, of which 84 percent is in the extended rainy period from June to September. The dry period lasts from October to February. The mean yearly highest and lowest temperatures are 260 °C and 140 °C, respectively, with a moisture content of 61.3% (Adardo, 2007). The laboratory investigation was undertaken at the Addis Ababa University, College of Veterinary Medicine and Agriculture, Microbiology Laboratory found in Bishoftu, and the Animal Health Institute found in Sebata, Ethiopia. In Bishoftu, approximately half of poultry a producer in Ethiopia are found in Bishoftu and surrounding with high percentage male farmers (Ebsa *et al*., 2019).



Source: created by Arch Map 10.2 software

Figure 2: Map of study area

3.2. Study Design

A cross-sectional study design was used to address the objectives of the study whereby samples were collected from randomly selected broiler and layer commercial poultry farms in Bishoftu town, central Ethiopia. A rapid questionnaire survey was also used in the poultry farms to acquire information pertaining to commonly used antibiotics and their availability, frequency of antibiotics usage and management practices on the respective farms. In addition, purposive visits were made to the poultry farms based on the type of production and size of the flock to address all farm environments.

3.3. Research Methods

3.3.1. Study Unit and Population

The study populations were both layer and broiler farms commercial poultry farms in Bishoftu, Ethiopia. The chickens belong to different age groups and selected randomly for sampling purposes.

3.3.2. Sample Type and Sampling Techniques

The sampling technique used in the present study was mainly random type. Accordingly, four types of samples were collected namely: Cloacal swabs, drinking water, feed and litter droppings. Cloacal swab samples were collected by gently introducing swab or cotton-tipped bacteriological swab into the chicken cloaca to the appropriate length and twirling to collect the sample. Subsequently, the swab was placed in a test tube with 10 ml of sterile buffered peptone water for transportation and pre-enrichment purpose. The sample from drinking water was taken with a sterile disposable syringe, and 1 ml of the sample was added to a test tube containing 9 ml of sterile buffered peptone water for enrichment and transportation. The other two types of samples, mainly feed samples and litter droppings were taken with sterile gloves and then transported to the lab with an ice box. Finally, in the lab, 25g of each was added independently to 225 ml of buffered peptone water for enrichment (ISO 6579, 2002).

3.3.3. Sample Size Determination

The sample size was determined on the basis of the formula described by Thrusfield (2018) with a 95% confidence interval. However, separate sample sizes were calculated for *Salmonella* and *Escherichia coli* independently as they do have variation in prevalence.

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

Where:

n = required sample size

1.96 = Z value at 95% confidence interval

p = expected prevalence/previously reported prevalence of the interested pathogen

d = desired precision, which is 0.05.

For the sample size calculation, the previous reported prevalence's for *salmonella* and *Escherichia coli* were 24.46% (Belachew *et al*., 2021) and 17.3% (Shimelis, 2022), respectively. As a result, the calculated sample size was 284 for Salmonella and 220 for Escherichia coli. However, the larger sample size (284) was used for both pathogens as the same sample was processed for the two pathogens.

Out of the total of 284 samples, 142 were collected from layer and another 142 from broiler commercial poultry farms to give equal opportunity for both types of farms. A total of 14 commercial poultry farms were randomly selected, of which 7 were layer poultry farms (2 large sized, 2 medium sized, and 3 small sized), and the remaining 7 were broiler (2 large, 2 medium, and 3 small). A total of 33 samples (30 Cloacal swabs, 1 drinking water sample, 1 feed sample and 1 litter drop) were collected from each large farm in both layer and broiler. Other 20 samples (17 Cloacal swabs, 1 drinking water sample, 1 feed sample, and 1 litter drop) were collected from each medium-sized farm in both farm types. Similarly, 12 samples (9 Cloacal swabs, 1 drinking water sample, 1 feed sample and 1 litter drop) from each small farm in both layer and broiler farm types were collected. Each farm was represented by one respondent, of whom 14 total respondents were selected for the rapid questionnaire survey.

3.4. Isolation and Identification of *Salmonella*

3.4.1. Cultural Methods

In this study, the cultural methods of Salmonella isolation were done according to the Global *Salmonella* isolation guideline of the World Health Organization (WHO) guideline and the protocol suggested by the International Organization for Standardization (ISO-6579, 2002) (Global *Salmonella* Survey, 2003; Quinn *et al*., 2004). This protocol recommends four steps for laboratory processes, including pre-enrichment in non-selective broth media, enrichment in selective enrichment broth media, plating on selective and differential solid media and confirmation by selected biochemical tests. The first step was pre-enrichment of *Salmonella* in non-selective liquid medium. It was done by using buffered peptone water (Oxoid, England) and incubating at 37°C for 24 hours. The second step was enrichment in selective broth media where Rappaport-Vassiliadis medium with soya (RVS broth) (Oxoid, England) was used in secondary enrichment. In general, 0.1 ml of the pre-enriched culture was transferred to a tube containing 10 ml of the RVS broth. The inoculated RVS broth was incubated at 41.5 °C for 24 hours. The third step was plating out the output from step two on selective solid media which was done by inoculating the cultures from secondary selective enrichment on selective and differential solid media such as XLD (Oxoid, England) and Brilliant Green Agar (Oxoid, England). XLD agar is used as both a selective and differential medium for *Salmonella* and other gram-negative bacteria. On XLD, *Salmonella* have a slightly transparent red/pink colony with a black center (Shalaby *etal*., 2022). Similarly, Brilliant Green Agar is also used as both selective and differential for *Salmonella* isolation in which the *Salmonella* colony is characterized as gray-reddish or pink and slightly convex (Kebede *et al*., 2016). Subsequent to recording the characteristics of a colony of *Salmonella* on all selective and differential mediums, the suspected colony was further cultured on a non-selective medium, Nutrient Agar, and finally subjected to selected biochemical tests for confirmation.

3.4.2. Biochemical methods

Biochemical tests such as Triple Sugar Iron (TSI) test, citrate utilization test, the methyl red test, and Vogues Proskauer (VP) test were conducted for further confirmation. The TSI test is used to assess the ability of bacteria to ferment glucose, lactose, and sucrose and their capability of forming hydrogen sulfide (H_2S) and other gases like Carbon dioxide (CO_2). In this case *Salmonella* is characterized by a red slant and yellow butt with the production of hydrogen sulfide and gas. Likewise, the citrate utilization test was used to evaluate the ability of bacteria to utilize sodium citrate to make use of it as a source of energy. In this test, *Salmonella* positive samples were characterized by a color change of the medium to blue appearance. *Salmonella* is positive for methyl red and negative for Vogues Proskauer in such a way that both tests use the same media. The difference comes from the reagents added after incubation and the principle of the reaction. The MR test is used to test the bacteria's ability to produce a large number of mixed acids as an end product of glucose fermentation and the VP test is used to determine the bacteria's ability to ferment glucose and produce acetyl methyl carbinol as an end product (Rasheed *et al*., 2014; Sannat *et al*., 2017).

3.4.3. Molecular Characterization

Molecular characterization of 26 *Salmonella* isolates confirmed by primary and secondar y methods was done at African Union Pan African Veterinary Vaccine Centre by using two different primers: a primer used to differentiate between *S. Gallinarum* and *S. pullorum* (Forward- SAL: 5' GACGTCGCTGCCGTCGTACC 3' and Reverse SAL: 5' TACAGCGAACATGCGGGCGG-3' and a primer used to detect general *Salmonella* (Forward-F.T: 5'-CCT GCG ACC ATC ATC CTG-3' Reverse_ F.T: 5'-TCA GCT CGC CAG TGG ATT-3') based on a protocol recommended by (Batista *et al*., 2016). A primer used to differentiate between *S. Gallinarum* and *S. pullorum* is based on bp to be generated at a time. During the process only a single product, either of 1,047bp (indicate *S. Gallinarum*) or 243bp (Indicate *S. pullorum*) expected to be generated. PCR process goes through steps: sample preparation/DNA extraction, PCR master mix preparation, thermocycling step, Agarose gel preparation for gel electrophoresis, agarose gel electroph oresis and reading of the results. All steps for detecting *salmonella* and differentiating *S. Gallinarum* and *S. pullorum* are the same and the only difference is the primer used for the two species.

DNA extraction/sample preparation:

Sample preparation for PCR was done according to a protocol suggested by (Batista et al., 2016) and a check list document provided by AU-PANVAC (Document no.AU PANV AC/306, C4.2). A pure colony of isolates was taken to AU-PANVAC molecular laborator y by sterile phosphate buffered saline (PBS) in a cryovial tube of 1.8ml with ice box. 560µl of prepared buffer AVL solution was pipetted in to 1.5ml microcentrifuge tube and add 140µ sample suspension and incubate for 10 min at room temperature. Then 560µl of 100% ethanol was added and mixed by vertexing. 630µl of the mixture was taken and transferred to QIAamp mini column of 2ml collection tube and centrifuged at 8000rpm for 1 min. The remain mixture (sample mixture) was added in to the same collection tube after removing the first one and centrifuged in the same way. After centrifuging, the collection tube was removed and 500µl of buffer AW1 was added and centrifuged at 8000 rpm for 1min, then add buffer AW2 of the same volume and centrifuge at 14000rpm for 3min. then the QIAamp mini column was placed in a new collection tube of 2ml and centrifuged at 15000rpm for 1min for column drying purpose. The spin column was then transferred to new microcentrifuge tube of 1.5ml and 60µl of buffer AVE was added directly into the membrane and centrifuged at 8000rpm from 1min.

PCR master mix preparation

PCR master mix was prepared based the protocol suggested by (Batista *et al*., 2016) and a check list document provided by AU-PANVAC (Document no. AU-PANVAC/QMS/QF /193/C4.311). a master mix for a single reaction was done as containing 10µl water, 2.5µl of Taq PCR buffer of 10x, 2.5µl of dNTPs mix 2mM, 2.5µl of FT-F 5µm and FT-R 5µm as fowl typhoid primers for detection of salmonella and 2.5µl of SAL-F 5µm and SAL-R 5µm as *Salmonella* enterica subsp. Enterica Serovar, Gallinarum biovars Gallinarum and Pullorum primers to differentiate between S. Gallinarum and S. pullorum, 0.125µl of Taq polymerase 5U/µl, 5µl of template DNA. This contains 25µl of final volume for 1reaction.

Finally, 23µ of the mix was transferred in to each PCR strip tube and 2µl of extract template DNA was added to the corresponding tubes and then subjected to thermocycler of different temperature of which initial denaturation at 94ºc for 3min, denaturation at 94ºC at 1min. for 25 cycles. Annealing at 63ºC for 30sec, extension at 72ºC for 60 sec. and final extension at 72ºC for 5min to have amplified fragment size of 587bp.

Agarose gel preparation: agarose gel was prepared by mixing 100ml TBE buffer and 1gram of agarose powder mixed well by heating in a microwave and nucleic acid staining dye was added after cooling of the solution. The molten agarose then poured into gel mold containing buffer solution and comb was placed to form pore to load sample. 2µl of loading dye and 5µl of sample (mixture) was added in to pore of agarose gel and gel electrophoresis was run for 1hr and followed by reading.

3.5. Isolation and Identification of *Escherichia coli*

3.5.1. Cultural Methods

All samples collected for *Escherichia coli* were processed at the College of Veterinary Medicine and Agriculture (CVMA) Microbiology Laboratory based on ISO 6579:2007 for isolation and identification, and finally confirmed by MALDI TOF at the Animal Health Institute found at Sebata. Based on ISO 6579:2007, the samples were processed through four steps: non-selective enrichment, selective enrichment, primary isolation and biochemical tests at the isolation and identification levels. For, the confirmation of *Escherichia coli* at Matrix assisted laser desorption/ionization technology (MALDI-TOF) level, the Extended Direct Transfer procedure was used (Zhang *et al.*, 2022). Confirmed *Escherichia coli* were finally subjected to antimicrobial sensitivity tests. The details of the procedure followed are mentioned as follows:

Non-selective enrichment:

This is a pre-enrichment step in which all samples were putted in sterile buffered peptone water (BPW) in such a way that the swab samples were putted in a test tube with 10 ml BPW, 1 ml of water in a test tube with 9 ml BPW, and 25 g of feed and litter in 225 ml BPW on the sample collection day and incubated at 37°C for 24 hrs.

Selective enrichment:

This is the second step in which modified tryptone soy broth (Oxford, England) was used for selective enrichment.1ml of pre-enriched sample was added to a tube containing 9ml of modified tryptone soy broth and incubated overnight at 41°C for 24 hours

Primary isolation:

Primary isolation was done by using selective and differential solid mediums: MacConke y agar (Oxford, England) and Eosin methylene blue (EMB) (Oxford, England). Selectivel y enriched samples in modified tryptone soya broth were cultured on MacConkey agar for the first step of primary isolation and incubated aerobically at 37°C for 24 hours. Following 24 hours of incubation, the plate was observed for typical colony characteristic s of *Escherichia coli*, which are likely to appear as pink colonies and lactose fermenters. Based on the characteristics of colony appearance, a single suspected colony was streake d on EMB agar for a second step of primary isolation and incubated at 37°C for 24 hours (Abu-Sini *et al*., 2023). Subsequent to incubation, the plate was observed for metallic sheen-forming colonies, and the metallic sheen-forming colonies were transferred to nutrient agar for biochemical tests.

3.5.2. Biochemical methods

The most appropriate biochemical tests conducted include Indole test, Methyl Red-Voges Proskauer (MR-VP) test, and Citrate Utilization Test. The tests were conducted by taking a pure colony from nutrient agar. The Indole test determines the ability of bacteria to split tryptophan molecules and produce Indole as an end product of the metabolic degradation of tryptophan amino acids, which has a positive reaction for *Escherichia coli*. *Escherichi a coli* reacts positively to the MR test but negatively to the VP test. MR is concerned with determining the ability of bacteria to produce large amounts of acid as an end product of glucose fermentation, and VP is used to determine the ability of bacteria to ferment glucose and produce acetyl methyl carbinol as an end product. A citrate utilization test was conducted to determine the ability of bacteria to degrade sodium citrate to use as an energy source, and *Escherichia* coli showed a negative reaction for this test (Geletu *et al*., 2022; Naser, 2016).

3.5.3. Matrix-assisted laser desorption and ionization technology (MALDI-TOF)

The Extended Direct Transfer (eDT) Procedure of MALDI TOF was conducted for more confirmation of *Escherichia coli* and *Salmonella* as protocol recommended by (Zhang *et al*., 2022) and Bruker MALDI Biotyper Manufacturer. A pure colony of *Escherichia coli* and *Salmonella* confirmed by primary and secondary confirmation methods was cultured on brain heart infusion agar. A single colony from BHI was taken by wooden application sticks and smeared as a thin film on a spot on a MALDI target plate. On the spot next to the smeared sample, add a bacterial test standard. 1 µl of 70% FA was added to each spot and allowed to dry at room temperature. After drying, 1µl of HCCA solution was added and again allowed to dry at room temperature. MALDI biotype measurements were performed at the end by using MALDI Biotyper Instrument.

3.5.4. Antimicrobial Susceptibility Test

An antimicrobial susceptibility test was conducted for confirmed *Escherichia coli* only as all samples processed for *Salmonella* were negative. Antimicrobial sensitivity test was done based on Kirby-Bauer disc diffusion method on Mueller-Hinton agar, and the interpretation was done according to the standard recommended by the National Committee for Clinical Laboratory Standards. Well-isolated colonies of *Escherichia coli* were cultured on Brain Heart Infusion agar. A bacterial suspension was prepared in a test tube with 5 ml of saline water and the turbidity of suspension was detected by the MacFarland densitometer to be 0.5 (Hudzicki, 2012). The prepared bacterial suspension of standard turbidity was swabbed on Muller-Hinton agar using a sterile cotton swab and allowed to dry for 15–30 minutes. Then selected antibiotics with known concentrations were putted on the swabbed plate at equal distance from each other and from the center. The swabbed plate with antibiotics was incubated at 37^0C for 24 hrs. and observed for zones of inhibition. The used antibiotics were Streptomycin (10µg), Sulphaamethoxazole -Trimethoprim (1.25/23.75µg), Amoxicillin and Clavulanic acid (30µg), Ciprofloxacin (5µg), Tetracycline (30µg), Ampicillin (10µg), Amoxacillin (10µg), Gentamycin (10µg) and Cefoxitin (30µg). The diameter of the zone of inhibition was measured using a digital caliper, and the resistance status of the isolates was decided based on the interpretive chart put out by the National Committee for Clinical Laboratory Standards in 2012.

3.3.4. Data Management and Analysis

The obtained data from each sample processing step and rapid questionnaire was recorded in an MS Excel spreadsheet and analyzed using R Studio version 3.6.2. All data were summarized by frequency table and Pearson's chi-square tests of R studio software were used for analyzing. Then the output of analyzed data were presented by using appropriate statistical methods, including graphs, percentages and P-value was used to identify the statistical significance of the results. The p-value ≤ 0.05 were considered as statistically significant and the p-value > 0.05 were considered as statistically not significant.

4. RESULTS

A total of 284 samples (142 from layer and 142 from broiler) were collected from 14 commercial poultry farms found in Bishoftu. In layer poultry farms, a total of 10 *Escheric hia coli* 6, 2, 2 from cloacal swab, litter and water samples, were isolated respectively. Ho wever, no isolate of *Escherichia coli* was found from feed in layer. From Broiler poultry farms *Escherichia coli* were isolated from 5 cloacal swab and 2 from feed samples. On the contrary, no *Escherichia coli* were isolated from both litter and water samples (Table 4). Of the 284 total sample processed for *Salmonella*, 38 samples (20 from layer and 18 from broiler) were confirmed by primary isolation and biochemical tests (TSI, MR-VP and Citrate utilization). The 26 isolates were subjected to MALDI-TOF and PCR. In both tests, all subjected isolates were found to be negative for *Salmonella*.

4.1. Biochemical test results of *Salmonella* and *Escherichia coli*

A total of 284 samples were processed for isolation and identification of both *Salmonella* and *Escherichia coli* based on respective primary isolation and biochemical tests. All collected samples were pass through selective enrichment on RVS, culturing on selective and differential solid medium (XLD, BGA) and biochemical tests (TSI, MR-VP, Citrate utilization test) step by step and 38 samples were confirmed to be *Salmonella*. The same sample size was subjected to selective enrichment on TSB, culturing on selective and differential media (MacConkey, EMB) and biochemical tests (Indole, MR-VP test, Citrat e Utilization test) for identification of *Escherichia coli* and 40 samples were found positiv e.

Table 3: Biochemical test results of *Salmonella* and *Escherichia coli*

Laboratory tests	Cultural/Biochemical tests results	MALDI-TOF results	PCR results	Overall confirmed total
Escherichia coli	40	17	-	17
Salmonella	38	negative	negative	negative

4.2. MALDI-TOF Confirmation test results of *Salmonella* and *Escherichia coli*

Of 38 isolates of *Salmonella* confirmed by primary and biochemical tests, only 26 (12 layer and 14 broiler) isolates and 40 *Escherichia coli* (25 layer and 15 broiler) were subjected to MALDI-TOF. An Extended Direct Transfer procedure were done and all suspected isolates to be *Salmonella* were confirmed to be negative and of 40 suspected *Escherichia coli,* 17 were confirmed as *Escherichia coli* and all isolates of *Salmonella* found to be negative and 17 isolates were confirmed as *Escherichia coli.*

4.3. PCR Test Results for *Salmonella* isolates

The total of 26 *Salmonella* isolates those were subjected to MALDI-TOF were further subjected to PCR test for molecular characterization at African Union Pan African Veterinary Vaccine Centre by using two different primers: a primer used to detect general *Salmonella* and a primer used to differentiate between *S. Gallinarum* and *S. Pullorum*. Consequently, all the isolates were found to be negative for the PCR test (Figures 3 and 4).

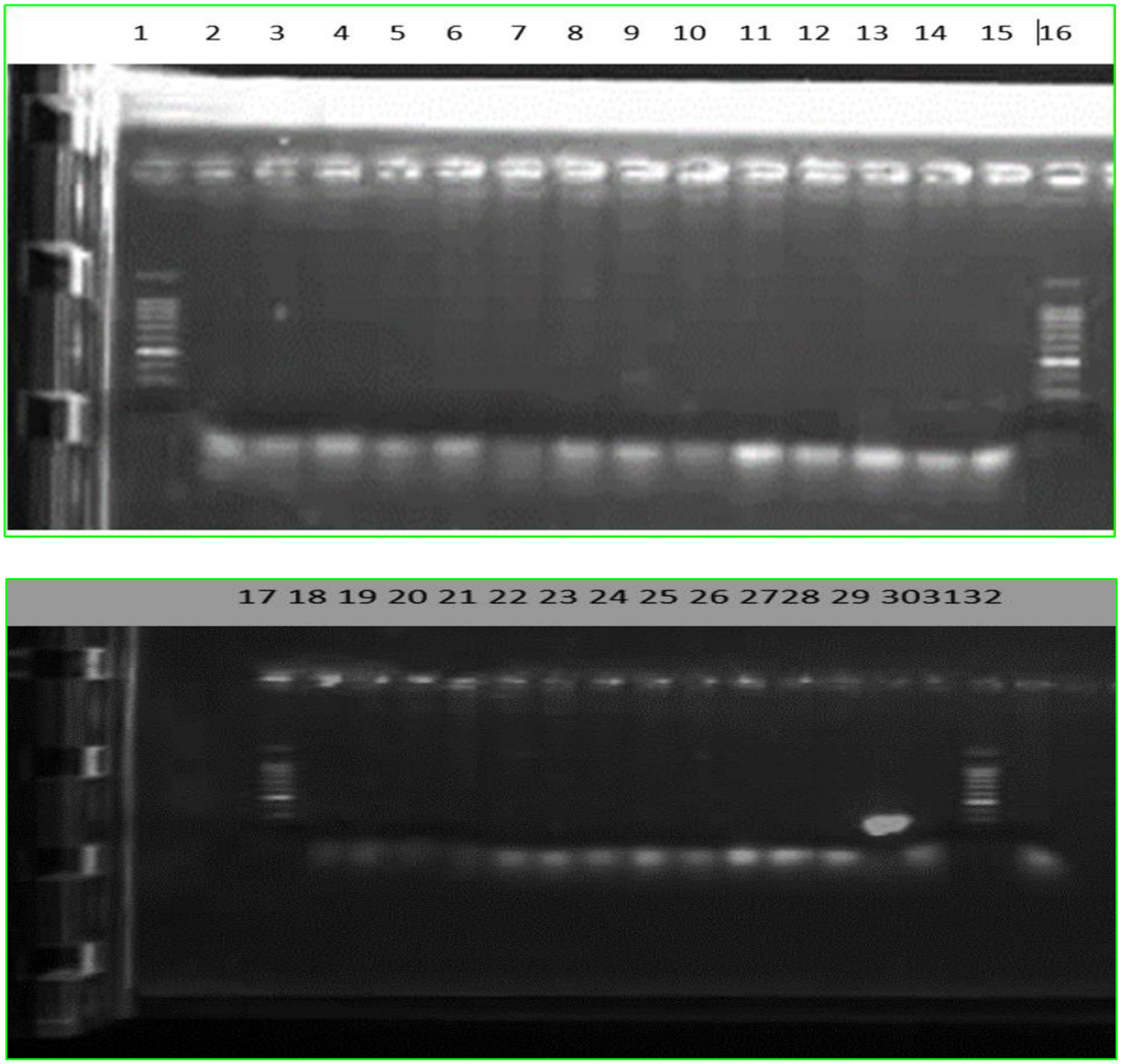


Figure 3: Gel picture of FT primer used for *Salmonella* detection.

Line 1, 16, 17 and 32 1-kb DNA ladder, line 30 positive control, 31 negative control. Line 3,5,9,13,23,24,25,26 and 28 are cloacal swab samples from layer poultry farm. Line 11,21 and 22 are litter, feed and water samples respectively from layer poultry. Line 2,4,6,7,8,10,12,15,18,19,27 and 29 are cloacal swab samples from broiler poultry farms. Line 14 and 20 are water and feed samples from broiler poultry farms. In the gel picture there is no product which aligns with the positive control and so there is no positive sample for *Salmonella*.

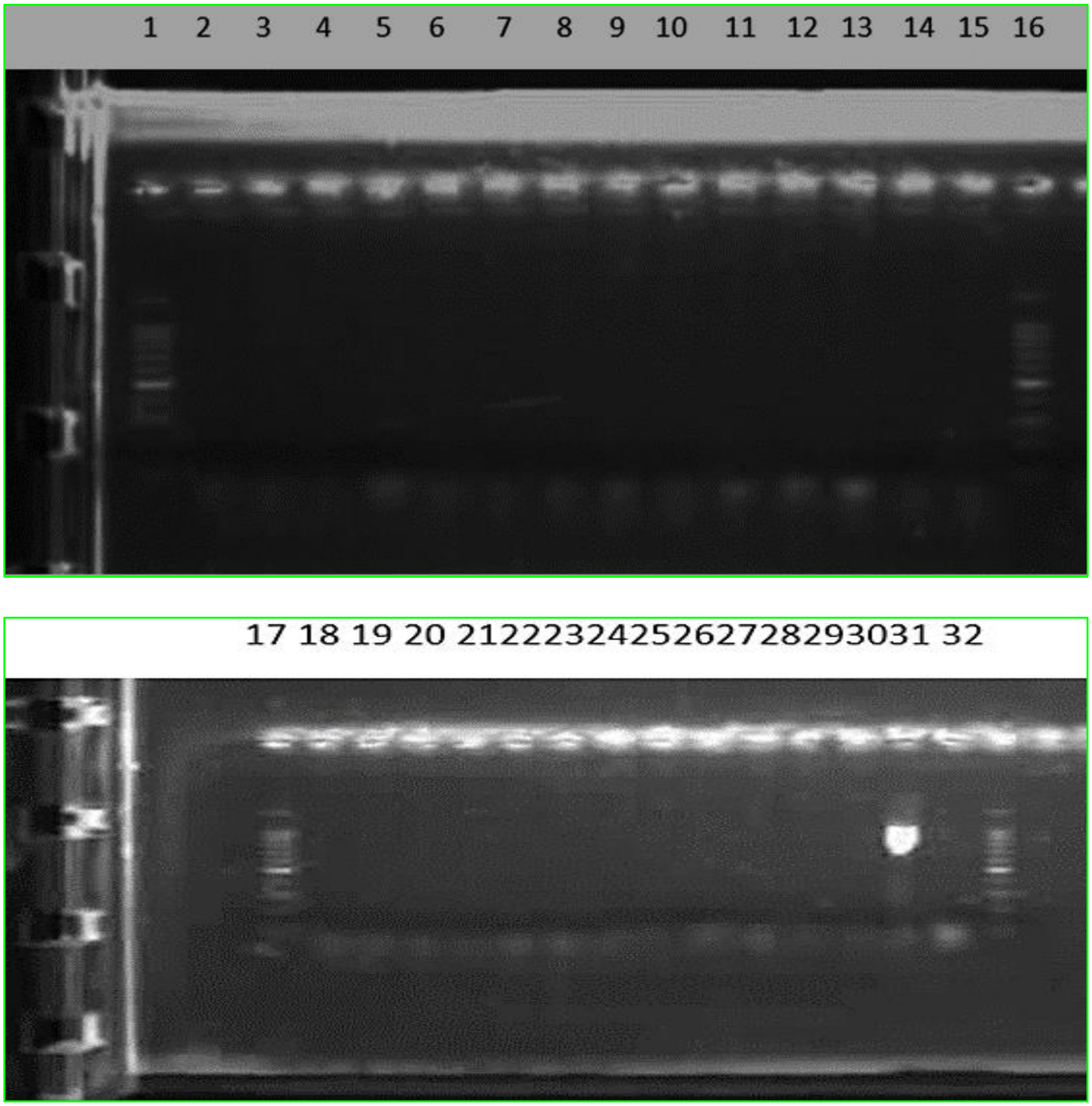


Figure 4: Gel picture SAL primer used to differentiate *S. Gallinarum* and *S. pullorum*. Line 1, 16, 17 and 32 1-kb DNA ladder, line 30 positive controls, 31 negative controls. Line 3,5,9,13,23,24,25,26 and 28 are cloacal swab samples from layer poultry farm. Line 11, 21 and 22 are litter, feed and water samples respectively from layer poultry. Line 2,4,6,7,8,10,12,15,18,19,27 and 29 are cloacal swab samples from broiler poultry farms. Line 14 and 20 are water and feed samples from broiler poultry farms. In the gel picture there is no product which aligns with the positive control and so there is no positive sample for both *S. Gallinarum* and *S. Pullorum*.

4.4. Antimicrobial Sensitivity Test Result of Escherichia *coli*

All confirmed isolates of *Escherichia coli* were subjected to Antimicrobial Sensitivity test at Animal Health Institute, Bacteriology laboratory. The result was interpreted as per the recommendation of CLSI, 2020. Nine types of antibiotics (Streptomycin (10mg), Sulp hamethoxazole-Trimethoprim (1.25/23.75mg), Amoxicillin and Clavulanic acid (30mg), Ciprofloxacin (5mg), Tetracycline (30mg), Ampicillin (10mg), Amoxicillin (10mg), Gent amycin (10mg) and Cefoxitin (30mg)) were use and their zones of inhibition were measured by Digital caliper for interpretation. Resistance status of Escherichia coli with respect of production type and sample type are indicated in table 5.

Table 4: Number and percentage of total positive sample with respect of total of each sample

Production type		No. and % of positive Swab	Total processed swab sample	No. and % of positive Litter	Total processed litter sample	No. and % of positive Feed	Total processed feed sample	No. and % of positive Water	Total processed water sample
Layer	Large	2(100%)	60	0/0%	2	0/0%	2	0/0%	2
	Medium	4(66.66%)	34	1/16.66%	2	0/0%	2	1/16.66%	2
	Small	0(0%)	27	1/50%	3	0/0%	3	1/50%	3
	Total	6(60%)	121	2/20%	7	0/0%	7	2/20%	7
Broiler	Large	5(71.43%)	60	0/0%/0%	2	2/28.57%	2	0/0%/0%	2
	Medium	0(0%)	34	0/0%/0%	2	0/0%/0%	2	0/0%/0%	2
	Small	0(0%)	27	0/0%/0%	3	0/0%/0%	3	0/0%/0%	3
	Total	5(71.43%)	121	0/0%/0%	7	2/28.57%	7	0/0%/0%	7
Total		11(64.71%)	242	2/11.76%	14	2/11.76%	14	2/11.76%	14

Table 5: Comparison of resistance profile of isolates from each sample type

Antimicrobial agents	**Resistance status of *E. coli* by % and No. from Layer**			**Resistance status of *E. coli* by % and No. from Broiler**	
	Swab	Water	Litter	Swab	Feed
Streptomycin (S-10)	4(66.67%) S 1(16.67%) I 1(16.67) R	0(0%) S 1(50%) I 1(50%) R	1(50%) S 1(50%) I 0(0%) R	1(20%) S 2(40%) I 2(40%) R	0(0%) S 0(0%) I 2(100%R
Sulfamethoxazole-Trimethoprim (1.25/23.75)	6(100%) S 0(0%) I 0(0%) R	0(0%) S 0(0%) I 2(100%) R	1(50%) S 0(0%) I 1(50%) R	3(60%) S 0(0%) I 2(40%) R	1(50%) S 0(0%) I 1(50%) R
Amoxicillin and Clavulanic acid (AMC-30)	4(66.67%) S 2(33.33%) I 0(0%) R	0(0%) S 2(100%) I 0(0%) R	2(100%) S 0(0%) I 0(0%) R	4(80%) S 1(20%) I 0(0%) R	1(50%) S 1(50%) I 0(0%) R
Ciprofloxacin (CIP-5)	4(66.67%) S 1(16.67%) I 1(16.67%) R	0(0%) S 1(50%) I 1(50%) R	2(100%) S 0(0%) I 0(0%) R	1(20%) S 0(0%) I 4(80%) R	0(0%) S 0(0%) I 2(100%) R
Tetracycline (TE-30)	4(66.67%) S 0(0%) I 2(33.33%) R	0(0%) S 0(0%) I 2(100%) R	1(50%) S 0(0%) I 1(50%) R	0(0%) S 0(0%) I 5(100%) R	0(0%) S 0(0%) I 2(100%) R
Ampicillin (AMP-10)	4(66.67%) S 1(16.67%) I 1(16.67%) R	0(0%) S 0(0%) I 2(100%) R	2(100%) S 0(0%) I 0(0%) R	3(60%) S 0(0%) I 2(40%) R	0(0%) S 0(0%) I 2(100%) R
Amoxicillin (AML-10)	0(0%) S 1(16.67%) I 5(83.33%) R	0(0%) S 0(0%) I 2(100%) R	0(0%) S 0(0%) I 2(100%) R	1(20%) S 2(40%) I 2(40%) R	0(0%) S 0(0%) I 2(100%) R
Gentamicin (CN-10)	6(100%) S 0(0%) I 0(0%) R	2(100%) S 0(0%) I 0(0%) R	2(100%) S 0(0%) I 0(0%) R	5(100%) S 0(0%) I 0(0%) R	0(0%) S 2(100%) I 0(0%) R
Cefoxitin (FOX-30)	3(50%) S 2(33.33%) I 1(16.67%) R	2(100%) S 0(0%) I 0(0%) R	2(100%) S 0(0%) I 0(0%) R	5(100%) S 0(0%) I 0(0%) R	1(50%) S 1(50%) I 0(0%) R

Keys: S= susceptible, I= intermediate, R= resistance

The overall result shows highest resistance to Amoxacillin (76.5%) and lowest resistance to Cefoxitin (5.9%). Of the total isolates, *Escherichia coli* showed highest resistance against Amoxicillin (90%) and Tetracycline (100%) in layer and Ciprofloxacin (85.7%) in broiler. Lowest susceptibility was recorded against Cefoxitin (10%) in layer and Sulfamethoxazole-trimethoprim (42.9%) among broiler poultry farms. In contrast no resistant isolates were found against Gentamicin and Amoxicillin-Clavulanic acid (0.0%). Whereas Cefoxitin, Gentamicin, and Amoxicillin-Clavulanic acid can be recommended as good antibiotics to use in farms as both layer and broiler showed highest degree of susceptibility (Table 6).

Table 6: Description of the antimicrobial resistance profile versus production type

Antimicrobials	**Resistance profile (no and %)**								
	Susceptible			Intermediate			Resistant		
	Total	Layer	Broiler	Total	Layer	Broiler	Total	Layer	Broiler
Streptomycin (S-10)	6(35.3)	5(50)	1(14.3)	5(29.4)	3(30)	2(28.6)	6(35.3)	2(20)	4(57.1)
Sulfamethoxazol Trimethoprim (1.25/23.75)	11(64.7)	7(70)	4(57.1)	0(0)	0(0)	0(0)	6(35.3)	3(30)	3(42.9)
Amoxicillin Clavulanic acid (AMC-30)	11(64.7)	6(60)	5(71.4)	6(35.3)	4(40)	2(28.6)	0(0)	0(0)	0(0)
Ciprofloxacin (CIP-5)	7(41.2)	6(60)	1(14.3)	2(11.8)	2(20)	0(0)	8(47.0)	2(20)	6(85.7)
Tetracycline (TE-30)	5(29.4)	5(50)	0(0)	0(0)	0(0)	0(0.0)	12(70.6)	5(50)	7(100)
Ampicillin (AMP-10)	9(52.9)	6(60)	3(42.9)	1(5.9)	1(10)	0(0)	7(41.2)	3(30)	4(57.1)
Amoxacillin (AML-10)	1(5.9)	0(0)	1(14.3)	3(17.6)	1(10)	2(28.6)	13(76.5)	9(90)	4(57.1)
Gentamicin (CN-10)	15(88.2)	10(100)	5(71.4)	2(11.8)	0(0)	2(28.6)	0(0)	0(0)	0(0)
Cefoxitin (FOX-30)	1(64.7)	5(50)	6(85.7)	5(29.4)	4(40)	1(14.3)	1(5.9)	1(10)	0(0)

4.5. Questionnaire Survey Results

A rapid questionnaire survey was done to identify the types of drugs most commonly used in farms and then to relate the resistance status of the isolates with antibiotic most commonly used in farms identified by questionnaire. Findings of rapid questionnaire survey showed that resistant isolates from both broiler and layer have previous treatment history with regard to Ciprofloxacin and tetracycline in both layer and broiler and additionally with Sulfamethoxazole-Trimethoprim in layer (Table 7).

Table-7: Previous treatment history of the resistant isolates to the antibiotics

Antimicrobial agents	**Layer**						**Broiler**			
	Swab		Water		Litter		Swab		Feed	
	With	Without	With	Without	With	Without	With	Without	With	Without
Streptomycin (S-10)	0	1	0	1	0	0	0	2	0	2
Sulfamethoxazole-Trimethoprim (1.25/23.75)	0	0	1	1	0	1	0	2	0	1
Amoxicillin and Clavulanic acid (AMC-30)	0	0	0	0	0	0	0	0	0	0
Ciprofloxacin (CIP-5)	1	0	1	0	0	0	2	2	1	1
Tetracycline (TE-30)	2	0	1	1	1	0	5	0	2	0
Ampicillin (AMP-10)	0	1	0	2	0	0	0	2	0	2
Amoxicillin (AML-10)	0	5	0	2	0	2	0	2	0	2
Gentamicin (CN-10)	0	0	0	0	0	0	0	0	0	0
Cefoxitin (FOX-30)	0	1	0	0	0	0	0	0	0	0

This table shows that resistant isolates from both broiler and layer have previous treatment history for ciprofloxacin and tetracycline and additionally for Sulfamethoxazol e-Trimethoprim in a layer, a significance test was done for these three antibiotics using x^2 test by R studio. The significance test for tetracycline shows a statistically significant between production type and resistant status (p=0.02) which shows there is a difference in resistance status between isolates from layer and broiler. The significance test between production type and treatment history (p=0.2), treatment history and resistance status (p=0.4), and resistance status and sample type (p=0.5) show statistically no difference is there. Significance test for ciprofloxacin shows no statistical difference is there in tests for treatment history and resistance status (p=0.5), resistance status and sample type (p=0.2) and there is a statistical difference in a test between production type and treatment history (p=0.03) and between production type and resistance status (p=0.02). A significance test for Sulfamethoxazole-Trimethoprim in all cases shows no statistically significant difference is there (Appendix 4).

Previous treatment history of the farms to antibiotics shows high usage of tetracycline, ciprofloxacin and sulfamethoxazole-trimethoprim in both layer and broiler farms, and no treatment history was recorded to streptomycin, amoxicillin and clavulanic acid, ampicillin, amoxicillin, gentamicin and cefoxitin in both farms. Therefore, tetracycline, ciprofloxacin and sulfamethoxazole are identified as the most commonly used antibiotics in both layer and broiler commercial poultry farms founds in the study area (Graphs 1 and 2)

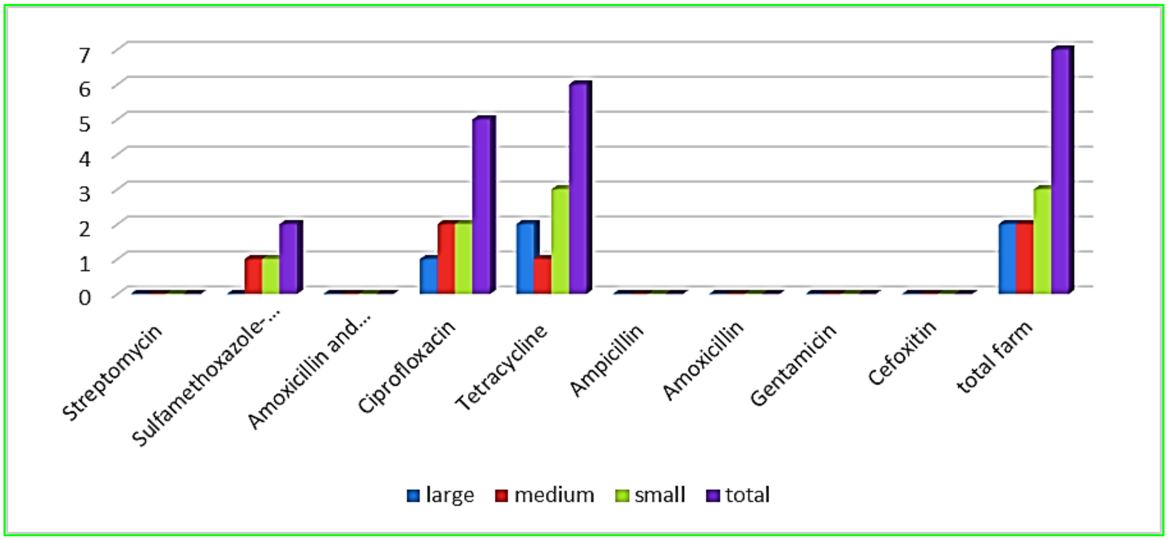


Figure 5: Antibiotic usage status in layer farms

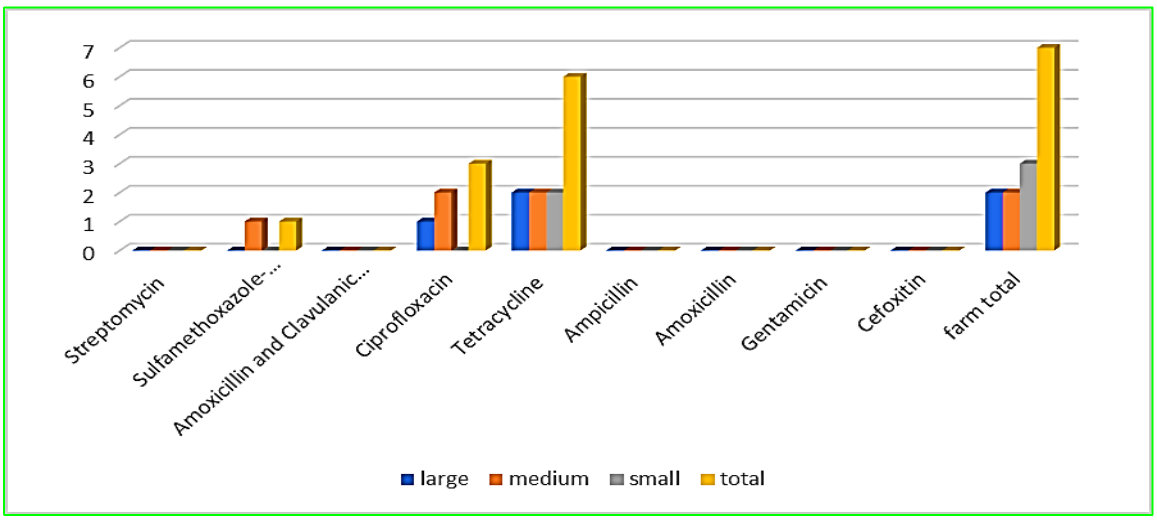


Figure 6: Antibiotic usage status in broiler farms

Antibiotic using status between farms shows the same using status tetracycline in both farm type and greater usage of ciprofloxacin and sulfamethoxazole-trimethoprim in layer poultry farms than broiler and the difference is statistically significant (p=0.0007) (Graph 7) (Appendix 4).

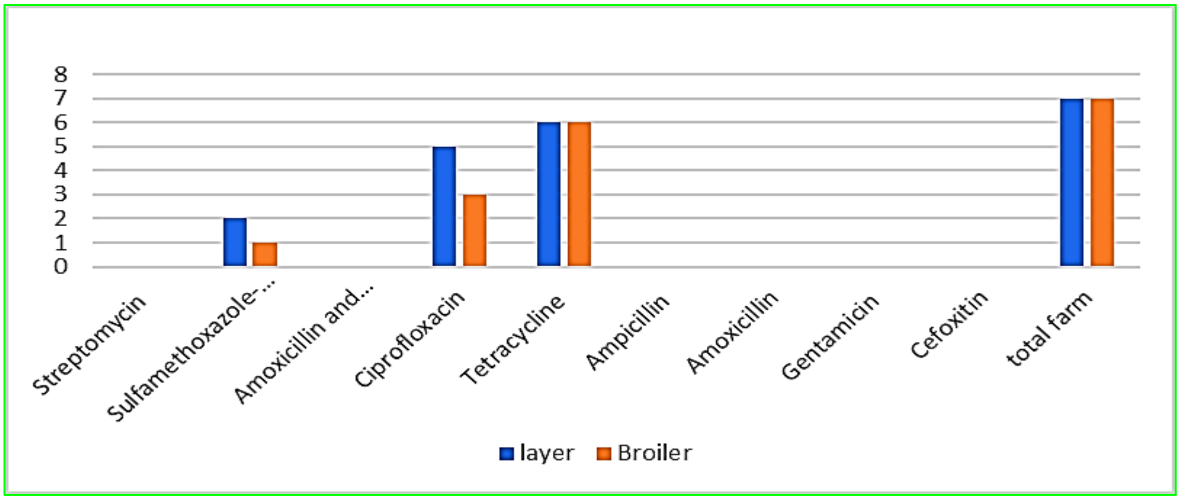


Figure 7: Comparison of antibiotic usage status between layer and broiler

Percentage of resistant *Escherichia coli* is greater in broiler than layer for all the most commonly used antibiotics and no comparison can be done between isolates from water, feed and litter as no isolates was found from water and litter in broiler and feed in layer (Figure 8).

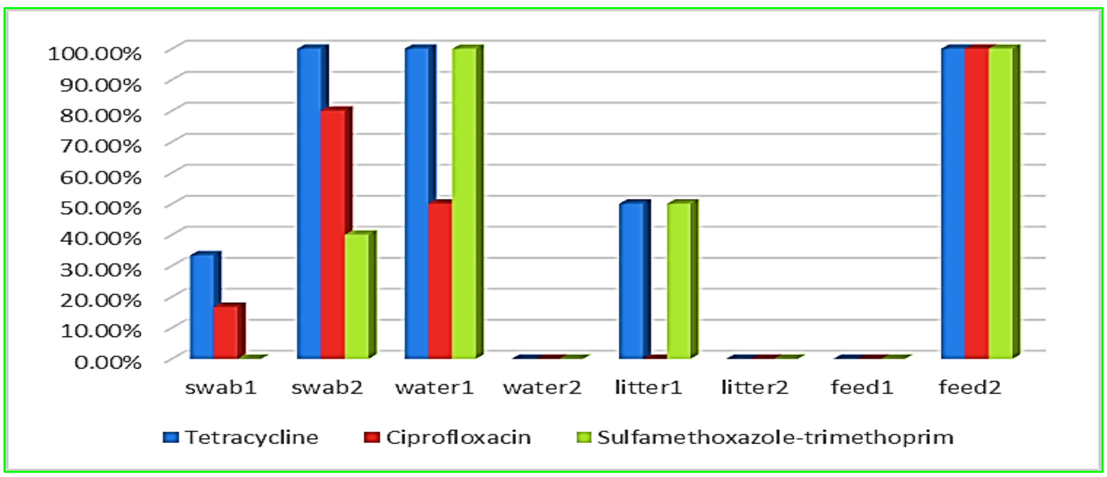


Figure 8: Resistance percentage comparison of resistant isolates to the most common antibiotics

Keys: Swab 1=cloacal swab from layer, Swab 2=cloacal swab from broiler, Water 1=water from layer, Water 2=water from broiler, Feed 1=feed from layer, Feed 2=feed from broiler, Litter 1=litter from layer, Litter 2=litter from broiler.

Out of the 14 farms visited for this study, 6 farms use antibiotics for preventive and treatment at a time and 5 farms use for all purposes (preventive, treatment and growth) and only 3 farms use only for treatment (Figure 7).

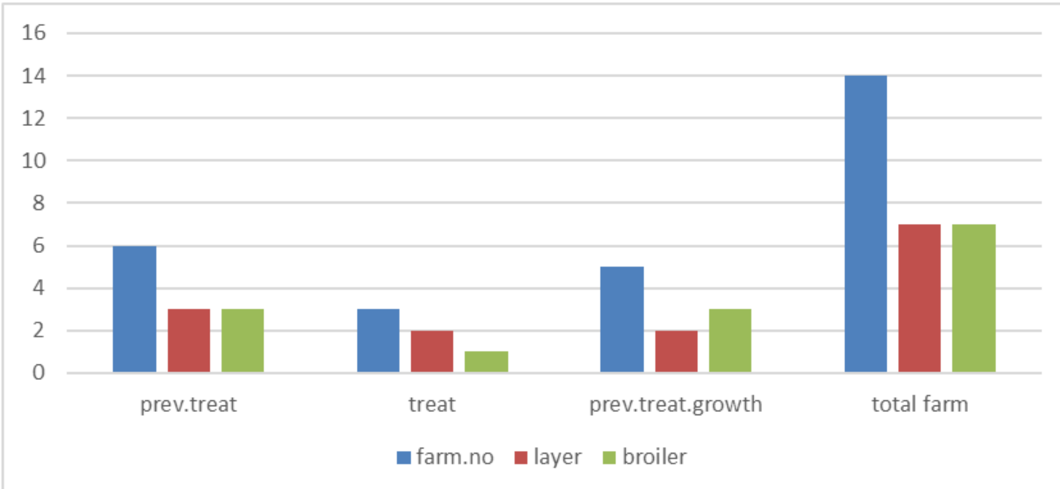


Figure 9: Antibiotic using purpose in farms

5. DISCUSSION

Colibacillosis and *Salmonellosis* mainly caused by *S. Gallinarum* and *S. pullorum* are considered as a major cause of morbidity and mortality in chickens and lead to significant economic losses to the poultry industry and are widely distributed in the world (Kim *et al.*, 2017; Batista *et al.*,2013). In response of this, detail investigation was carried out in this study on isolation and confirmation of *Salmonella* and *Escherichia coli* by MALDI-TOF and Molecular Characterization of *Salmonella* as MALDI-TOF and molecular confirmation of microbials is becoming popular. Assessment of antibiotic usage of the selected farm were conducted and antimicrobial sensitivity test was done for the confirmed isolates (Assoumy *et al.*, 2021; Singhal *et al.*, 2015). In this study, only isolates of *Escherichia coli* was found (17/284) which shows 6% prevalence and it is less prevalent than indicated as 17.3% by (Shimeles, 2022). No isolates were confirmed to be positive for *Salmonella* by both MALDI-TOF and PCR from both layer and broiler poultry farms in disagreement with a prevalence of *Salmonella* reported as 14.6% by (Eguale, 2018) and 24.46% by (Belachew *et al.*, 2021). High prevalence of *Salmonella* has also reported from other countries in contrast of this study: Uganda (Odoch *et al.*,2017), Nigeria (Fagbamila *et al.*,2017), Burkina Faso (Kagambèga *et al.*,2013) Gambia (Dione *et al.*,2011) as 21%, 23%, 55% and 67% respectively. Again in contrast of this study, (Sannat *et al.*, 2017) and (Batista *et al.*, 2016) identify and molecularly characterize *S. Gallinarum* and *S. pullorum* as a main cause of fowl typhoid and pullorum disease in poultry.

The results of this study on isolation and confirmation of *Escherichia coli* shows that 17(6%) were found to be positive from 284 total samples of cloacal swab, feed, litter and water, which is more or less comparable with reports of (Aseffa *et al.*, 2011), (Hassanain *et al.*, 2012) and 12.5% (Urji *et al.*, 2005) as 11.5%, 11.4% and 12.5% respectively as a little percentage difference may be raised from a little sample size difference. But cannot be comparable with reports of (Ashwani *et al.*, 2014) as 15.2%.

According to the result of this study, more isolates were found from layer 7.04% which is not comparable with report of (Gedeno *et al.*, 2022) as 43.10%. This report shows high percentage of *Escherichia coli* from approximately 2times less sample size (58 total sample from chicken layer) and low percentage is found by this study from large sample size (142 sample from layer). A total of 242 cloacal swab sample were collected from both layer and broiler and percentage of positivity of samples to *Escherichia coli* is found to be 4.55% which is highly disagree with reports found by (Kiiti *et al*., 2021) as 100% positivity from 402 cloacal swab sample from both layer and broiler. 121 cloacal swab was collected independently from broiler and prevalence of *Escherichia coli* indicates 5(4.13%) incomparable with reports of (Ibrahim *et al*., 2020) 51.8% , (Geidam, 2012) 60% and (Kim, M. and Dong, 2021) 100%. 14 total litter dropping sample were collected from 14 randomly selected layer and broiler farms and 2 samples were found to be positive with 14.28% and 0% prevalence in layer and broiler respectively. Even though production type as layer and broiler did not mentioned, high prevalence of *Escherichia coli* from litter dropping is reported by (Langata *et al.*, 2019) as 57%.

In case of isolates from broiler, *Escherichia coli* from feed sample shows 100% resistance against antibiotics: Streptomycin, Ciprofloxacin, Ampicillin, Amoxicillin in agreement with (Ngai *et al*., 2021) in case of Ampicillin which is reported as 71% resistance as the percentage difference can be as a result of difference in sample size and is disagree in case of Ciprofloxacin reported as 0% resistance of *Escherichia coli* by the same author and found to be 100% by present study from broiler poultry feed. Total isolates of *Escherichia coli* (10) from layer showed highest resistance to Amoxicillin (90%) and Tetracycline (50%) can be related with 40% and 37% resistance respectively (Kim *et al*., 2022). Current study founds *Escherichia coli* as having no resistance to both Gentamicin and Amoxicillin-Clavulanic acid. In contrast of this study, (Kim *et al*., 2022) have reported 21.1% resistance against Amoxicillin-Clavulanic acid. Even though there are consistence results of this study with previous reports, attention should be given on those inconsistence results to dig out why it happens.

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed that *Escherichia coli is a* bacterial infectious disease-causing agent and prevalent in the study area and the commonly used antibiotics administered for the chickens are resistant. The conventional and necessary laboratory activities were done to isolate and confirm *Salmonella and Escherichia coli* by MALDI TOF and PCR and finally no isolate was confirmed as *Salmonella*. However, *Escherichia coli* were predominantly isolated from samples from layer farm and isolates from swab sample have high percentage. Generally, *Escherichia coli* resistance profile indicates highest resistance to Amoxicillin, and least to Cefoxitin but no resistance was observed against Gentamicin and Amoxicillin-clavulanic acid. Independently, *Escherichia coli* from broiler showed highest resistant against Tetracycline and Ciprofloxacin and Amoxicillin in layer. But no resistant isolates were found against Gentamicin, Amoxicillin-clavulanic acid and Cefoxitin in both farm type and in broiler respectively. The antimicrobial sensitivity test revealed highest resistance against tetracycline and ciprofloxacin in layers and amoxicillin in broilers. The rapid questionnaire on the use of antibiotics indicated indiscriminate utilization for prevention-treatment, for treatment and for treatment-prevention-growth purposes in a farm which might attribute to higher level of antimicrobial resistance.

Based on this conclusion the following recommendations are forwarded.

1. Farms are need to improve farm management and poultry biosecurity measures in order to minimize or eliminate the pathogens studied and or other challenging factors to the farms.
2. Detail and molecular investigation should be done on antibiotics showing variance resistance profile for different production type and sample type
3. Strong public awareness creation about rational usage of antimicrobials and having of antibiotics administration's policy/regulation is crucial.
4. The test agreement /discrepancy among the diagnostic tests should be furtherly evaluated.

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8. ANNEXES

Annex 1: List of Media

MacConkey agar:

Typical formula:	*Composition*	
Gram Per Litter	Peotone	17.00,
	Agar	*13.5*
	Lactose	*10.00*
	Sodium Chloride	*5.00*
	Bile Salt	*1.50*
	Protease peptone	3.00
	Neutral Red	0.03
	Crystal violet	0.001

Preparation:

- Suspend 50 grams of the medium in one liter of distilled water.
- Mix well until a uniform suspension is obtained.
- Heat with frequent gentle agitation and boil for one minute.
- Sterilize in autoclave at 121º C (15 lbs. sp) for 15 minutes.
- Cool to 45 °C, and pour into Petri dishes.
- Allow the plates to solidify and place them upside down to avoid excessive moisture in the surface of the medium.

Nutrient Agar:

Typical formula:	*Composition*	
Gram Per Litter	Gelatin Peptone	5.00
	Yeast extract	2.00
	Lactose Bacteriological Agar	15.00
	Sodium chloride	*5.00*
	Final PH	7.4 ± 0.2 at 25ºC
	Distilled water	1 liter.

Preparation:

- Suspend 28 grams of the medium in one liter of distilled water.
- Mix well and leave to stand until the mixture is uniform.
- Heat with gentle agitation and boil for one or two minutes, or until completely dissolved.
- Sterilize by autoclaving at 121°C for 15 minutes. Dispense in sterile Petri dishes.

Modified Tryptone Soya Broth:

Typical formula:	***Composition***	
Gram Per Litter	Pancreatic digest of casein	17.0
	Papaic digest of soybean meal	3.0
	Dipotassium hydrogen phosphate	4.0
	Glucose	*2.5.*
	Bile Salts	1.5

Preparation:

- Dissolve 33 grams of the powder in one liter of distilled water.
- Heat with frequent agitation to completely dissolve the medium and sterilize by autoclaving at 121°C for 15 minutes.
- Cool to 45-50°C and add 2 vials of NCM4040-0.5* Novobiocin Supplement, each reconstituted using 5mL of sterile deionized/RO water.

Mueller Hinton agar

Typical formula:	***Composition***	
Gram Per Litter	Beef Infusion	300.00
	Casein hydrolysate	17.50
	Starch	1.50
	Bacteriological Agar	17.00
	Final PH:	7.4 ± 0.2 at 25ºC
	Distilled water	1 liter

Preparation:

- Suspend 38 grams of medium in one liter of distilled water.
- Bring to boil to dissolve the medium completely.

- Sterilize by autoclaving at 121°C for 15 minutes.

Triple Sugar Iron Agar:

Typical formula:	***Composition***	
Gram Per Litter	Peptone Mixture	20.00
	Lactose	10.00
	Sucrose	10.00
	Sodium Chloride	5.00
	Beef Extract	3.00
	Yeast Extract	3.00
	Glucose	1.00
	Ferrous Ammonium Citrate	0.30
	Sodium Thiosulphate	0.30
	Phenol Red	0.024
	Bacteriological Agar	12.00
	Final pH:	7.4 ± 0.2 at 25ºC
	Distilled water	1 liter.

Preparation:

- Suspend 65 grams of the medium in one litter of distilled water.
- Bring to boil to dissolve completely. Mix well and sterilize by autoclaving at 121º C for 15 minutes.
- Distribute in sterile tubes and cool it in a slanted position, as to obtain butts of 1.5–2 cm depth.

Simmons citrate agar

Typical formula:	***Composition***	
Gram Per Litter	Ammonium dihydrogen phosphate	1.0
	Dipotassium phosphate	1.0
	Sodium chloride	5.0
	Magnesium sulphate	0.2
	Agar	15.0
	Bromotymol blue	0.08

Preparation:

- Suspend 24.2g of the powder in 1litre of distilled water and mix well.
- Heat with frequent agitation and boil to completely dissolve the powder.
- Sterilize by autoclaving at 1210C for 15 minutes, then dispense in tubes in slant form.

MR-VP Medium

Typical formula:	***Composition***	
Gram Per Litter	Peptone	7.0
	Glucose	5.0
	Phosphate buffer.	5.0

Preparation:

- Suspend 17g in 1 liter of distilled water.
- Mix well, distribute in a final container and sterilize by autoclaving at 1210C for 15 minutes.

Eosin Methylene blue Agar

Typical formula:	***Composition***	
Gram Per Litter	Peptone	10.0
	Lactose	10.0
	Dipotassium hydrogen phosphate	2.0
	Eosin Y	0.4
	Methylene blue	0.0065
	Agar	15.0.

Preparation:

- Suspend 35.7g in 1 liter of distilled water.
- Bring to boil to dissolve completely.
- Sterilize by autoclaving at 1210C for 15 minutes.
- Cool to 600C and shake the medium in order to oxidize the methylene blue (restore its blue color and to suspend the precipitate which an essential part of the medium).

Tryptone Soya Broth

Typical formula:	*Composition*	
Gram Per Litter	Casein enzyme hydrolysate	10.0
	Sodium chloride	5.0
	Final pH (at 25°C)	7.5±0.2

Preparation:

- Suspend 15 grams in 1000 ml distilled water.
- Heat if necessary to dissolve the medium completely.
- Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Rappaport-Vassiliadis medium with soya:

Typical formula:	*Composition*	
Gram Per Litter	Soya peptone	4.5
	Sodium chloride	8.00
	Potassium dihydrogen phosphate	0.60
	Dipotassium phosphate	0.40
	Magnesium chloride, hexahydrate	29.00
	Malachite green	0.036.

Preparation:

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

Xylose Lysine Deoxycholate Agar (XLD)

Typical formula:	*Composition*	
Gram Per Litter	Yeast extracts	3.0
	l-lysine hydrochloric acid	5.0
	Xylose	3.75
	Lactose	7.5
	Sucrose	7.5
	Sodium Thiosulphate	6.8
	Ferric ammonium citrate	0.8
	Phenol red;	0.08
	Agar	15.0
	Sodium chloride	5.0

Preparation:

- Suspend 53grams in one liter of distilled water.
- Heat with frequent agitation until the medium boils. Do not over heat.
- 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.
- PH: 7.4 + 0.2 at 25 ℃.

Brilliant Green Agar

Typical formula:	*Composition*	
Gram Per Litter	Peptone	5.0
	Tryptone	5.0
	Lactose	10.0
	Sucrose	10.0
	Sodium chloride	5.0
	Phenol red	0.080
	Brilliant green	0.0125
	Agar	15.0.

Preparation:

- Suspend 58.09 grams in 1000 ml purified /distilled water.
- Then Heat to boiling to dissolve the medium completely.

- Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Avoid overheating.
- Cool to 45-50°C.
- Mix well and pour into sterile Petri plates. Final pH (at 25°C): 6.9±0.2.

Buffered Peptone Water

Typical formula:	***Composition***	
Gram Per Litter	Enzymatic digest of casein	10.0
	Sodium chloride	5.0
	disodium phosphate dodecahydrate	9.0
	potassium dihydrogen phosphate	9.0

Preparation:

- Suspend 20 grams in 1000ml of distilled water.
- Mix well and distribute into universal bottle of suitable capacity to obtain the portions necessary for the test and sterilize in autoclave at 121 ℃ for 12 minutes.
- Final PH is 7.0 + 0.2 at 25℃.

Brain Heart Infusion agar

Typical formula:	***Composition***	
Gram Per Litter	HM infusion	12.5
	BHI powder	5.0
	Protease peptone	10.0
	Dextrose or glucose	2.0
	Sodium chloride	5.0
	Disodium phosphate	2.50
	Agar	15.0.

Preparation:

- Suspend 52.0 grams into 1litre of distilled water, heat to boiling and sterilize by autoclaving at 15 lbs. pressure (121ºC) for 15 minutes.
- Cool it at 45-50ºC and pour it on sterilized Petri dishes.

Annex 2: List of Biochemical Tests

Triple Sugar Iron Agar (TSI) test

Principle: TSI agar is used to determine whether a Gram-negative rod utilizes glucose and lactose or sucrose fermentative and forms hydrogen sulphide (H2S). TSI contains 10 parts lactose: 10 parts sucrose: 1 part glucose and peptone. Phenol red and ferrous sulphate serves as indicators of acidification and H2S formation, respectively. The formation of CO2 and H2 is indicated by the presence of bubbles or cracks in the agar or by separation of the agar from the sides or bottom of the tube. The production of H2S requires an acidic environment and is indicated by blackening of the butt of the medium in the tube.

Procedure: With a straight inoculating wire, touch the top of a well isolated colony. Inoculate TSI by first stabbing through center of the medium to the bottom of the tube and then streaking the surface of the agar slant. Leave the cap on loosely and incubate the tube for 18-24 hours at 35oC in an incubator.

Result interpretation:

1. Alkaline slant/no change in the butt (K/NC) = Glucose, lactose and sucrose non-utiliser
2. Alkaline slant/acid butt (K/A) = Glucose fermentation only
3. Acid slant/acid butt (A/A), with bubble or cracks = Glucose, sucrose, and/or lactose fermenter with gas production.
4. Alkaline slant/acid butt (K/A), black at the butt = Glucose fermentation only and Hydrogen sulphide production.

Citrate Utilization test:

Principle: The test organism is cultured in a medium which contains sodium citrate, an ammonium salt and the indicator bromothymol blue. Growth in the medium is shown by turbidity and a change in color of the indicator from light green to blue, due to alkaline reaction following citrate utilization.

Procedure: Inoculum is streaked over the slant of Simmons citrate agar in a tube and incubated for 24-48 hrs.

Result interpretation: Growth on the slant and change in color to blue of the medium indicates positive result.

Indole Test

Principle: Indole test is performed to determine the ability of the organism to split tryptophan molecule into Indole. Indole is one of the metabolic degradation products of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and deaminating tryptophan with the production of Indole, Pyruvic acid and ammonia.

Procedure: Inoculate Tryptone broth with the test organism and incubate for 24hrs at 37OC.Then add 15 drops of Kovacs reagent down the inner wall of the tube.

Interpretation: Development of bright red color at the interface of the reagent and the broth within seconds after adding the reagent is indicative of the presence of Indole and is a positive test

Methyl Red Test

Principle: To test the ability of the organism to produce and maintain stable acid end products from glucose fermentation and to overcome the buffering capacity of the system.

Procedure: Inoculate the MR/VP broth with a pure culture of the test organism and incubate at 35OC for 48 to 72 hrs. Add 5 drops of MR reagent to the broth.

Result interpretation: Positive result is red (indicating pH below 6). Negative result is yellow (indicating no acid production).

Voges Proskauer Test

Principle: To determine the ability of the organisms to produce neutral end product acetyl methyl carbinol (acetoin) from glucose fermentation.

Procedure: Inoculate pure culture of the test organism into VP broth and incubate for 24 hrs at 37OC. Aliquot 1 ml of the broth to a sterile test tube and add 0.6ml of VP reagent (5% Alpha-Naphthol and ethanol) followed by 0.2ml of VP reagent (40% Potassium Hydroxide). Shake the tube gently to expose the medium to atmospheric oxygen and allow the tube to remain undisturbed for 10 to 15 minutes.

Interpretation: Positive: Pinkish red color at the surface of the medium. Negative: Yellow color at the surface of the.

Annex 3: List of Reagents and Chemicals

McFarland turbidity standard preparation

Solution 1

1.175 grams of $BaCl_2 \cdot 2H_2O$ was measured. 50 ml of distilled water was added and mixed well. Make up to 100 ml with distilled water.

Solution 2

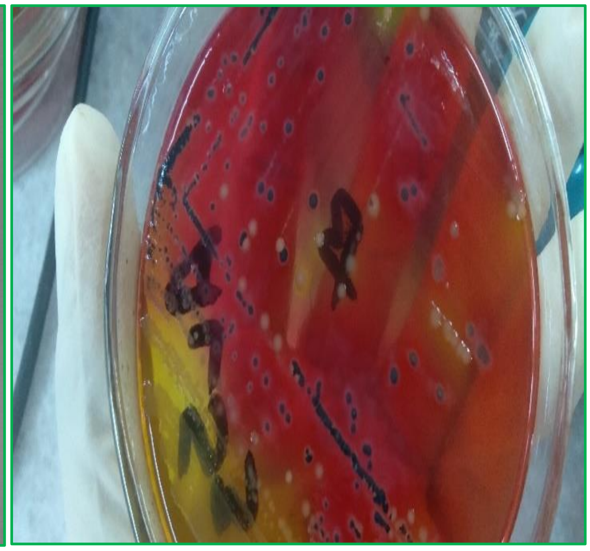
99 ml of distilled water was put in to the flask. 1 ml of concentrated H_2SO_4 was added in to the flask contained distilled water. 0.5 McFarland turbidity=99.5 ml "solution 2" (1% H_2SO_4 + 0.5 ml "solution 1" 1.175% $BaCl_2$).

Reagents used in biochemical tests

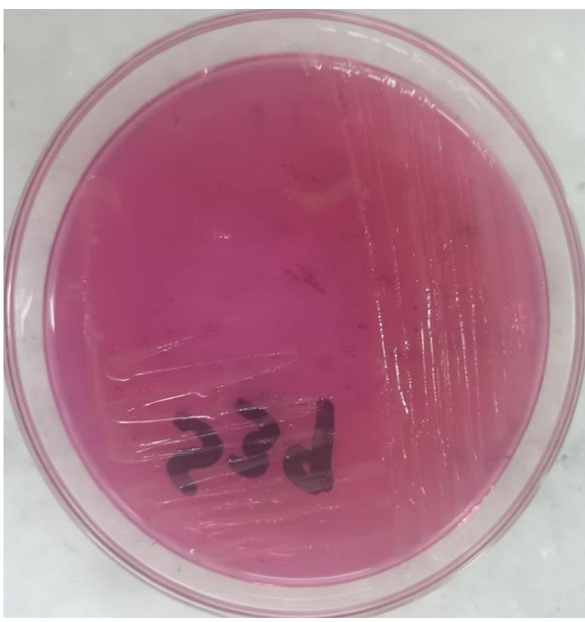
- Methyl red reagent: used for methyl red test
- ∝- naphthol, strong alkali (40% KOH), and atmospheric oxygen
- Indole Kovac's reagent

Annex 4: Supportive Pictures for *Salmonella* Identification

Salmonella on RVS (color change of the Medium showed by turbidity)

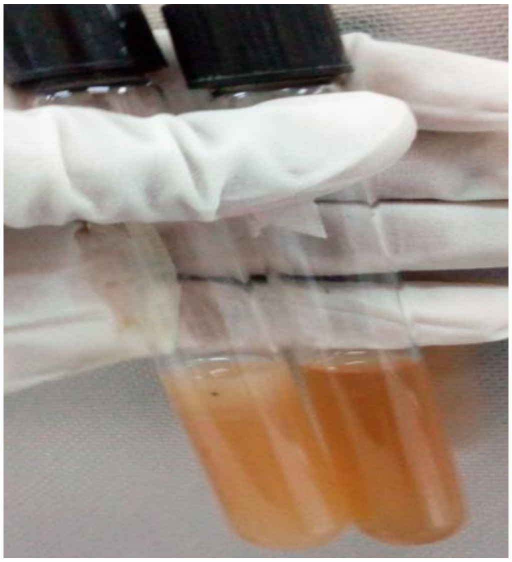


Salmonella on XLD (pink/red colony with black center)

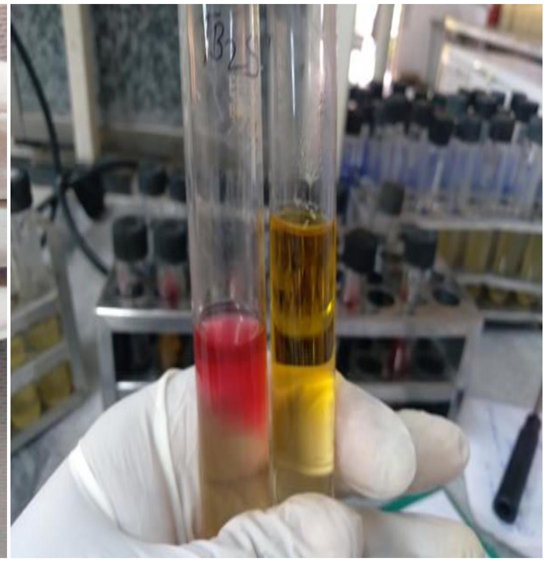


Salmonella on BGA (pale pink colony)

Citrate test for *Salmonella* (+ve, color change of the medium to blue)



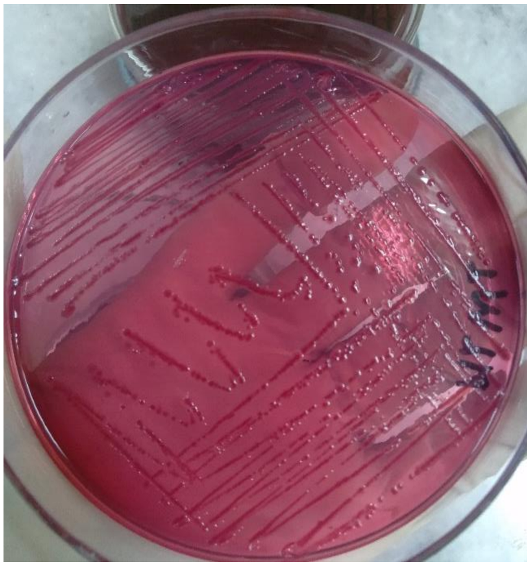
VP test (-ve for *Salmonella*)



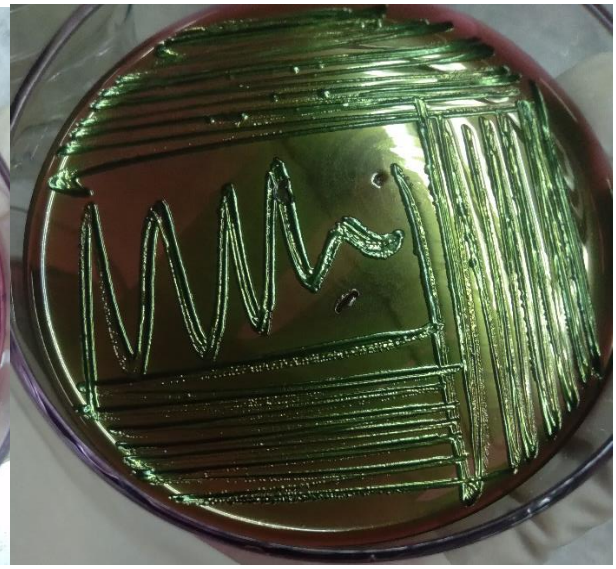
MR test (+ve for *salmonella*)

TSI test (Red/yellow, H_2S, Gas)

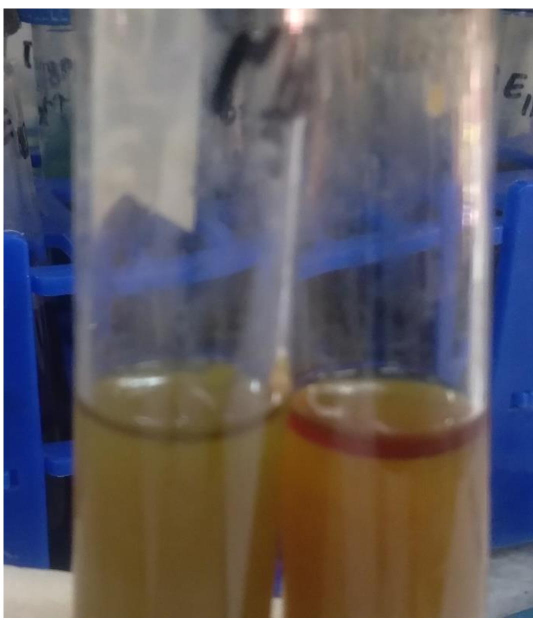
Annex 5: Supportive Pictures for Escherichia coli identification



E. coli on MacConkey

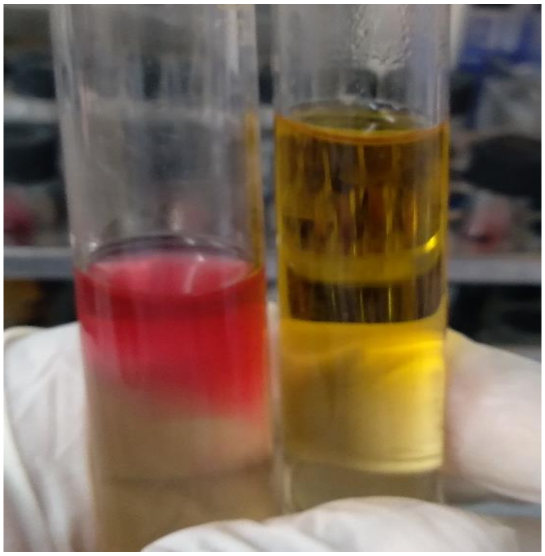


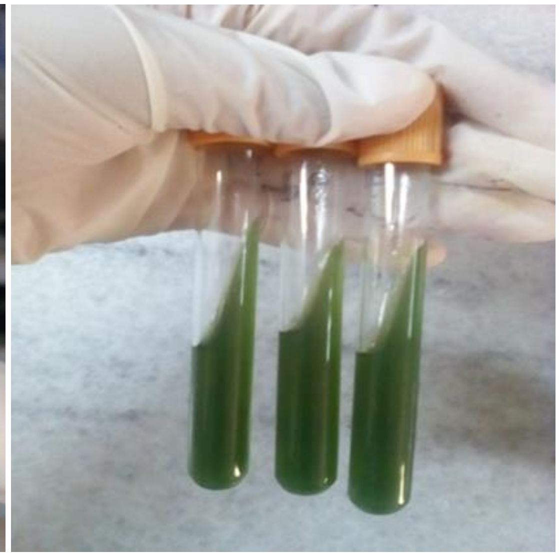
E.coli on EMB



Indole test (+ve)

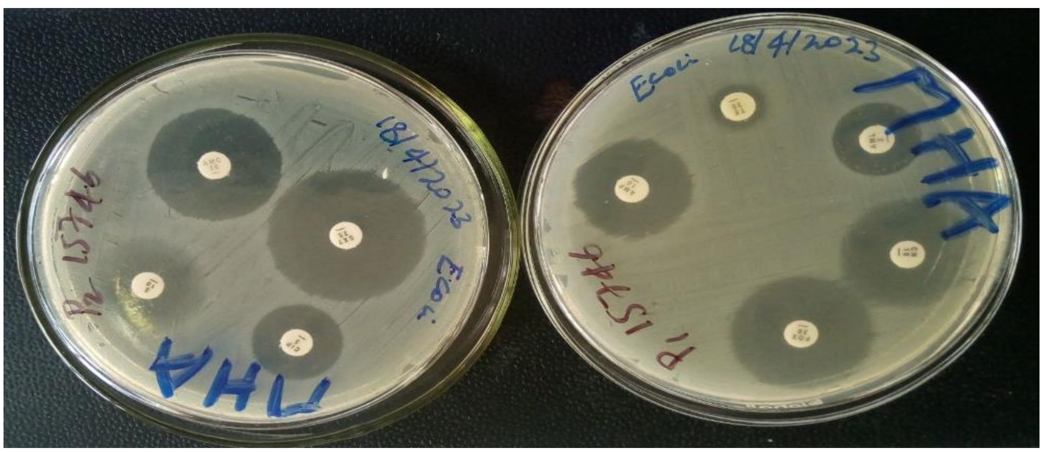
VP test (-ve)

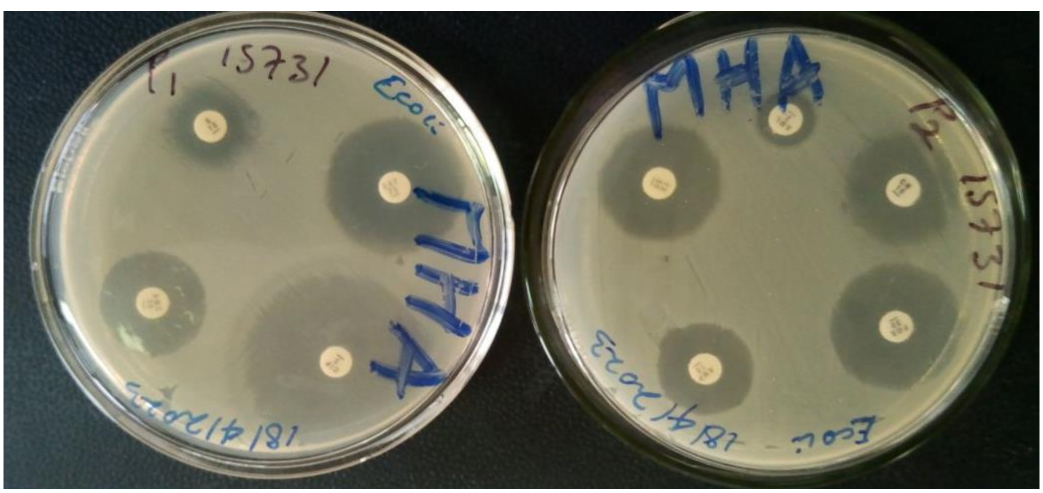




MRtest (+ve) Citrate utilization test (-ve)

Annex 6: Antimicrobial Sensitivity Test Plate





9. APPENDIXES

Appendix 1: MALDI TOF test for *Salmonella*

Bruker MALDI Biotyper Identification Results

BRUKER

Run Info:

Run Identifier: 230422-1249-1011027865
Comment: E.coli Abdi AMR Surviellaince and Bilisuma Salmonellosis Bishoftu,2023
Operator: Admin@MBT-WIN10
Run Creation Date/Time: 2023-04-22T14:22:01.580
Number of Tests: 27
Type: Standard
BTS-QC: passed
BTS-QC Position: C3:0
Instrument ID: 8604832.05381
Server Version: 4.1.100 (PYTH) 174 2019-06-158_01-16-09

Result Overview

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A1 (+++) (A)	15775 (Standard)	Proteus mirabilis	2.01	Proteus mirabilis	1.99
A2 (+) (C)	15763 (Standard)	Proteus mirabilis	1.91	Shigella dysenteriae	1.84
A3 (+++) (A)	15778 (Standard)	Proteus mirabilis	2.17	Proteus mirabilis	2.11
A4 (+++) (A)	15777 (Standard)	Proteus mirabilis	2.09	Proteus mirabilis	2.08
A5 (+++) (A)	15769 (Standard)	Proteus mirabilis	2.26	Proteus mirabilis	2.22
A6 (-) (C)	15764 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
A7 (+++) (A)	15771 (Standard)	Proteus mirabilis	2.25	Proteus mirabilis	2.24

Result overview table--continued on next page

Report created at 2023-05-25T11:05:37 **Research Use Only** Page 1 of 58

Result overview table--continued from previous page

Sample Name	Sample ID	Organism (best match)	Score Value	Organism (second-best match)	Score Value
A8 (+) (A)	15778 (Standard)	Proteus mirabilis	1.94	Proteus mirabilis	1.92
A9 (+++) (A)	15774 (Standard)	Proteus mirabilis	2.09	Proteus mirabilis	2.08
A10 (-) (C)	15421 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
A11 (-) (C)	15358 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
A12 (-) (C)	15371 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B1 (-) (C)	15336 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B2 (-) (C)	15324 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B3 (+++) (A)	15334 (Standard)	Escherichia coli	2.41	Escherichia coli	2.37
B4 (-) (C)	15335 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B5 (+++) (A)	15339 (Standard)	Escherichia coli	2.29	Escherichia coli	2.18
B6 (+++) (A)	15340 (Standard)	Escherichia coli	2.24	Escherichia coli	2.23
B7 (-) (C)	15346 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B8 (-) (C)	15362 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B9 (-) (C)	15325 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B10 (-) (C)	15326 (Standard)	**no peaks found**	0.00	**no peaks found**	0.00
B11 (+) (B)	s1 (Standard)	Escherichia coli	1.71	**No Organism Identification Possible**	1.67

Result overview table--continued on next page

Appendix 2: Result report from AHI for Escherichia coli

AHI

TEST RESULT REPORT FORM

Customer name: Bilisuma Abebe
Contact: phone : 0946953545, email : bilisuma abebe004@gmail.com
Sampling Place: Region: Oromiya, District: Bishoftu Town
Kebele: Bishoftu Town

Reference No. SR/RE/186/2023
Test Report No. 186/2023

Date reported: 04-05-2023

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed
15723-15762	Bacterial Isolate	Not Defined	15/10/2022	11/04/2023	16/04/2023
					17/04/2023

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	40	17	23	0	E.coli isolation and identification	MALDI-TOF	AHI-TM-BACL-003*

*Accredited Test (AHI is accredited for tests marked *)

Opinions and Interpretations

-Out of the 40 bacterial isolate suspected Escherchia coli, 17 were positive for the test and the rest were negative.

Verified by:
Olani Abebe

Approved by:

Dr.Getnet Abie Mekonnen
Deputy Director General

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NOTE: the results shown on this test report only refers the number of samples tested and the respective customer not for other purpose. AHI is responsible for all the information provided on this report, except for the information provided by the customer.

		Approved by: Abera Kabede
Effective Date: 2022.07.25	Document Type: FRM	Document Number: AHI-FRM-GEN-025-8A

PO BOX 04: Sebeta Town, Ethiopia +251113380894/95/96/97 Fax: +251113380220 Website: http://www.ahi.gov.et/

ATTACHMENT

Samples type: Bacterial Isolate- Species: Not Defined

List of Positive Samples: E.coli isolation and identification / MALDI-TOF

SR No	Lab Code	Sample's Id	Result	Location
1	15729	E15	Positive	Bishoftu
2	15730	E16	Positive	Bishoftu
3	15731	E17	Positive	Bishoftu
4	15733	E20	Positive	Bishoftu
5	15734	E21	Positive	Bishoftu
6	15735	E23	Positive	Bishoftu
7	15736	E25	Positive	Bishoftu
8	15737	E27	Positive	Bishoftu
9	15739	E30	Positive	Bishoftu
10	15746	E49	Positive	Bishoftu
11	15748	E53	Positive	Bishoftu
12	15749	E55	Positive	Bishoftu
13	15750	E56	Positive	Bishoftu
14	15751	E58	Positive	Bishoftu
15	15752	E60	Positive	Bishoftu
16	15753	E62	Positive	Bishoftu
17	15754	E63	Positive	Bishoftu

List of Negative Samples: E.coli isolation and identification / MALDI-TOF

SR No	Lab Code	Sample's Id	Result	Location
1	15723	E1	Negative	Bishoftu
2	15724	E3	Negative	Bishoftu
3	15725	E5	Negative	Bishoftu
4	15726	E6	Negative	Bishoftu
5	15727	E8	Negative	Bishoftu
6	15728	E11	Negative	Bishoftu
7	15732	E19	Negative	Bishoftu
8	15738	E28	Negative	Bishoftu
9	15740	E34	Negative	Bishoftu
10	15741	E37	Negative	Bishoftu
11	15742	E38	Negative	Bishoftu
12	15743	E43	Negative	Bishoftu
13	15744	E44	Negative	Bishoftu
14	15745	E47	Negative	Bishoftu
15	15747	E51	Negative	Bishoftu
16	15755	E65	Negative	Bishoftu
17	15756	E66	Negative	Bishoftu
18	15757	E68	Negative	Bishoftu
19	15758	E69	Negative	Bishoftu
20	15759	E71	Negative	Bishoftu
21	15760	E72	Negative	Bishoftu
22	15761	E74	Negative	Bishoftu

NOTE: the results shown on this test report only refers the number of samples tested and the respective customer not for other purpose. AHI is responsible for all the information provided on this report, except for the information provided by the customer.

		Approved by: Abera Kabede
Effective Date: 2022.07.25	Document Type: FRM	Document Number: AHI-FRM-GEN-025-8A

Appendix 3: Questionnaire

A questionnaire prepared to collect information on commonly used Drugs /antibiotics in broiler and layer poultry farms selected for sample collection.

Part I: A questionnaire to be filled by interviewer

1. Size of poultry flock:

A. large

B. medium

C. small

2. Production type of poultry farm:

A. Broiler

B. Layer

Part II: Questionnaire to be filled by selected personnel from each farm selected for sample collection.

1. What is your educational level

A. Primary school

B. High school

C. College

D. University

E. Other______________________________

2. What is your professional area?

A. Veterinarian

B. Veterinary laboratory technology

C. Animal health/animal science

D. Veterinary pharmacy

E. Other____

3. What is your responsibility in this farm?

A. Farm owner

B. Farm manager

C. Attendant

D. Farm veterinarian

E. Other____

4. Would you mention the type of diseases/symptoms you have experienced in your farm

A.____

B.____

C.____

D.____

6. Types of drugs/antibiotics you use most commonly

____

7. For what type of purpose, you use antibiotics/drug

A. Growth promoter

B. Preventive/prophylaxis

C. Treatment

8. Types of antibiotics you use for growth promoter

____

9. Types of antibiotics you use for preventive/prophylaxis

____

10. Types of antibiotics you use for treatment

____

11. From where did you buy/get antibiotics/drugs

A. From veterinary pharmacy

B. From human pharmacy

C. From individuals

D. Other__________________________

12. Who orders/prescribes the drugs/antibiotics

____

13. Did you think the antibiotics you are using are effective?

____

14. When do you use antibiotics?

A. As needed for treatment

B. When disease outbreak occurred only

C. Other_________________________

15. At what interval do you use antibiotics

____

16. Who gives the drugs to the poultry?

A. Owner of the farm

B. Farm manager

C. Attendant

17. Is he/she professional?

A. Yes

B. No

Appendix 4: Data analysis table

p-value analysis table for drug usage difference between layer and broiler

Number of cases in table: 10

Number of factors: 2

Test for independence of all factors:

Chisq = 40, df = 16, p-value = 0.0007786

Chi-squared approximation may be incorrect

p-value analysis table for Tetracycline

treatment history Vs resistance statusof Tetracycline

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 0.4628, df = 1, p-value = 0.4963

Chi-squared approximation may be incorrect

production type Vs treatment history of Tetracycline

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 1.5867, df = 1, p-value = 0.2078

Chi-squared approximation may be incorrect

sample type Vs resistance status of Tetracycline

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 2.3311, df = 3, p-value = 0.5066

Chi-squared approximation may be incorrect

production type Vs resistance status of Tetracycline

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 4.958, df = 1, p-value = 0.02597

Chi-squared approximation may be incorrect

p-value analysis table for Ciprofloxacin

treatment history Vs resistance ofCiprofloxacin

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 1.0878, df = 2, p-value = 0.5805

Chi-squared approximation may be incorrect

Production type Vs treatemet history ofCiprofloxacin

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 4.408, df = 1, p-value = 0.03577

Chi-squared approximation may be incorrect

sample typeVs resis. status ofCiprofloxacin

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 8.541, df = 6, p-value = 0.2011

Chi-squared approximation may be incorrect

produc.type, Vs resis.status ofCiprofloxacin

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 7.268, df = 2, p-value = 0.02641

Chi-squared approximation may be incorrect

p-value analysis table for Sulfamethoxazole-Trimethoprim

treat.history, Vs resis.status of Sulfamethoxazole-Trimethoprim

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 0.21465, df = 1, p-value = 0.6431

Chi-squared approximation may be incorrect

produc.type, Vs treat.history of Sulfamethoxazole-Trimethoprim

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 1.5867, df = 1, p-value = 0.2078

Chi-squared approximation may be incorrect

sample type Vs resis.status of Sulfamethoxazole-Trimethoprim

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 5.456, df = 3, p-value = 0.1413

Chi-squared approximation may be incorrect

produc.type,resis.status of Sulfamethoxazole-Trimethoprim

Number of cases in table: 17

Number of factors: 2

Test for independence of all factors:

Chisq = 0.29805, df = 1, p-value = 0.5851

Chi-squared approximation may be incorrect

Appendix 5: Ethical clearance report

Ethical clearance form approved by Ethics Review Committee of CVMA

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የእንስሳት ሕክምናና
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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

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

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

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

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

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

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

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

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

Getachew Terefe (DVM, PhD)
Chairman

Signature

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Please quote Our Ref. No. When replying

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