ISOLATION AND IDENTIFICATION OF *SALMONELLA* AND *E. COLI* FROM THE ENVIRONMENTAL SOURCES IN POULTRY FARMS IN HARAR AND HARAMAYA TOWN, EASTERN ETHIOPIA

MSc THESIS

BARENTO ABRAHIM

OCTOBER, 2021

HARAMAYA UNIVERSITY, HARAMAYA

Isolation and Identification of *Salmonella* and *E. coli* from the Environmental Sources in Poultry Farms in Harar and Haramaya Town, Eastern Ethiopia

A Thesis submitted to the college of Veterinary Medicine

Postgraduate Program Directorate

HARAMAYA UNIVERSITY

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE IN VETERINARY MICROBIOLOGY

Barento Abrahim Abdullahiy

OCTOBER, 2021

Haramaya University, Haramaya

HARAMAYA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

I hereby certify that I have read and evaluated this Thesis entitled "Isolation and identification of *Salmonella* and *E. coli* from the environmental sources in poultry farms in Harar and Haramaya town, Eastern Ethiopia" prepared under my guidance by Barento Abrahim. I recommend that it will be submitted as fulfilling the thesis requirement.

Bruk Abraha (DVM, MSc, Assoc. Professor) ______________ _______________

Major Advisor Signature Date

Yitagel Terafe (DVM, MSc, Assoc. Professor) ______________ ________________

Co-Adisor Signature Date

As members of the Board of Examiners of the MSc Thesis Open Defense Examination, I certify that I have read and evaluated the Thesis prepared by Barento Abrahim **"Isolation and identification of *Salmonella* and *E.coli* from the environmental sources in poultry farms in Harar and Haramaya town, Eastern Ethiopia",** we recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Veterinary Microbiology. I recommend that the thesis be accepted as fulfilling the thesis requirement for the Degree of Master of Science in Veterinary Microbiology.

Sisay Girma (DVM, MSc Assi. Professor) ______________ _______________

Chair Person Signature Date

Jilalu Kamal (MSc Micro Assi. Professor) ______________ _______________

Internal Examiner Signature Date

Eyob Hirpa (DVM, MSc, Assoc. Professor) ______________ _______________

External Examiner Signature Date

Final approval and acceptance of the Thesis is contingent upon the submission of its final copy to the Council of Postgraduate Program (CPGP) through the candidates College Graduate Committee (CGC).

DEDICATION

I dedicate this work to my advisors.

****

I dedicate this work to my family.

****

I dedicate this work to artist **Hacaaluu Hundeessaa Bonsa**. Let God keep his soul in Heaven.

STATEMENT OF AUTHOR

First, I declare that this thesis is my hardship time work during covid-19 time and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfilment of the requirements for an advanced (**MSc**) degree at Haramaya University, College of Veterinary Medicine and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: **Barento Abrahim**

Signature: ______________

College of Veterinary Medicine

Date of Submission:______________________

BIOGRAPHICAL SKETCH

Barento Abrahim Abdullahiy was born in Damota *kebele*, Haramaya *woreda* of East Hararge Zone, Oromia on August 11, 1993 G.C. He attended his primary, secondary, high school and preparatory in Bate and Haramaya schools respectively until 2013 G.C. He then joined Wollega University, College of Medical and Health Sciences, School of Veterinary Medicine in June 25, 2016 G.C and graduated with B.Sc. degree in Veterinary Laboratory Technology (VLT) in June 25, 2016 G.C. Then after he worked as animals research expert at a capacity of unit head for three years in Haramaya University Laboratory Management Directorate until he joined Haramaya University for his MSc study. He joined school of graduate studies of Haramaya University, Haramaya College of veterinary medicine in May 2, 2021 G.C to pursue MSc degree in veterinary microbiology. Currently, Barento is married and has two children.

ACKNOWLEGMENTS

First of all, praise is to Allah (the lord of the universe) for giving me the inner strength and ability to accomplish this study.

The work in this study would not have been possible without the help and support from many people and institutions.

I would like to express my utmost gratitude to my major advisor Dr. Bruk Abraha and co-advisor Dr. Yitagele Terefe for their support throughout the work of this thesis.

I am very much grateful to the laboratory technician Mrs. Beza Mulugeta and Mr. Dereje Regasa who helped me during laboratory work at Haramaya University. I am also grateful to Dr. Jemal Yousuf for the support and financial aid through the project “CADGED and EXCAMS”.

Finally, I express my thanks to all my family members, especially to my wife Hamdiya Mohammed Ali (Haadha Fizaan and Milki) for her support and encouragement.

LIST OF ACRONYMS AND ABBREVIATIONS

BGA	Brilliant Green Agar
CDC	Center of Disease Control
CSA	Central Statistical Agency
DCA	Deoxycholate-Citrate Agar
EMB	Eosin Methylene Blue
E. coli	*Escherichia coli*
FAO	Food and Agricultural Organization
FT	Fowl Typhoid
GPV	Gross Production Value
HDADB	Haramaya district Agricultural Development Bureau
HCLFO	Harar city Livestock and Fisheries Office
HIV	Human Immunodeficiency Virus
H_2S	Hydrogen Sulphide
ISO	International Standard Organization
MDR	Multi Drug Resistance
MIU	Motility Indole Urea
MRVP	Methyl Red/Voges-Proskauer
NA	Nutrient Agar
NB	Nutrient Broth
PCR	Polymerase Chain Reaction
PD	Pullorum Disease
SLT	Shiga Like Toxin
SIM	Sulphide Indole Motility
Spp.	Species
TSI	Triple Sugar Iron
TTB	Tetrathionate Broth
TVCs	Total Viable Counts

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ABSTRACT

Isolation and Identification of *Salmonella* and *E. coli* from the Environmental Sources in Poultry Farms in Harar and Haramaya Town, Eastern Ethiopia

The present study was conducted from October 2020 to October, 2021 with the objectives of isolation and identification of *Salmonella* and *E. coli* from environmental sources in poultry farms in Harar and Haramaya towns, eastern Ethiopia. A total of 52 samples comprising 48 from 12 poultry farms in Harar and 4 samples from 1 poultry farm in Haramaya were collected based on random sampling. Standard cultural and biochemical methods were used to isolate both *Salmonella* and *E. coli*. SPSS version 20.0 software (IBM) was used for the analyses of data. The overall prevalence of *E. coli* and *Salmonella* from the samples was 50% and 59.61%, respectively. The analysis of risk factors showed that *Salmonella* occurrence was significantly influenced ($p<0.05$) by manure disposal types, farm cleaning practices, and educational status of farm workers. *E. coli* prevalence was significantly associated ($p<0.05$) with risk factors such as poultry farm systems, in that it was higher in traditional (85.5%) than modernized one (28.1%). The prevalences of *E. coli* and *Salmonella* spp based on sample types were 69.2% and 38.4% in water; 46.2% and 84.6% in litter; 46.2% and 69.23% in feed; and 38.5% and 46.2% in swab samples of poultry houses. Generally, the study revealed that contact surfaces in the poultry farms are the potential sources for the both *E. coli* and *Salmonella* and large proportions of farm environments are contaminated with the tested bacterial species. The isolated bacteria could have a significant public health consequence due to direct transmission from the environment as well as infected poultry and their products through the food chain. Thus, in the control of infectious diseases, it is important to give high priority in controlling dissemination of bacterial pathogens in the environments and poultry farms operational units should be based on modernized systems and in a way that is suitable for cleaning and disinfecting.

Key words: *E. coli, feed, poultry farm, litter, Salmonella, swabs, water.*

1. INTRODUCTION

Ethiopia has an estimated chicken population of 59.42 million with native chicken breeds representing 85.68%, and the remaining 7.32 and 7.0% are hybrid chickens and exotic breeds, respectively (CSA, 2019). Inadequate knowledge about poultry production, limited feed resources, low productivity of indigenous chicken breeds, high prevalence of diseases and predators are the main constraints among of backyard poultry production in developing countries including Ethiopia (Tadelle *et al*., 2003; Wong *et al*., 2017; Bayesa, 2021).

Poultry is one of the fast-growing sources of food in the world today. Thus, the giving a target for poultry farms regarding to the keeping and tactical managements are very important starting from feeding up to the harvesting of the output (Hemen *et al.,* 2012). The major problem facing the poultry industry is the large-scale accumulation of wastes including manure and litter which may pose disposal and pollution problems unless environmentally and economically sustainable management technologies are evolved (Bolan *et al.,* 2010).

According to Das *et al*. (2005), poultry farm is hampered by a number of factors, of which infectious diseases are considered as the major factor causing 30% mortality of chicken per year, among which diarrhoea causing bacterial diseases such as *Escherichia coli* infections and Salmonellosis are the common one. *Escherichia coli* are a common pathogen for commercial poultry causing colibacillosis all over the world (Barens and Gross, 1997). It is a major cause of respiratory and septicemic diseases in broiler chicken causing mortality less than 5% and morbidity over 50% but in layer it affects the reproductive tract resulting failure of egg productivity and fertility. Salmonellosis is one of the most important bacterial diseases in poultry causing heavy economic losses through mortality and reduced meat and egg production (Haider *et al.,* 2004).

The avian intestines have been considered as a reservoir of potential *Salmonella* and *E. coli* with zoonotic potential that could be transferred directly from birds to humans. While, *E. coli* is a commensal bacterium in humans and animals and has a wide range of hosts, it is commonly present in the environment and considered an indicator of fecal contamination of food and water (Mude *et al*., 2017). Poultry products are some of the sources for transmitting

food borne pathogens to humans with 40% of the clinical cases attributed to the consumption of egg and other poultry products (Hossain *et al.,* 2011; Behravesh *et al.,* 2014).

Several studies have demonstrated that the sources of microbial infections in poultry include contaminated feeds, drinking water, utensils, personnel, human wastes, rodents, and hatchery related unhygienic activities (Okonko *et al.,* 2010). Moreover, it has been reported that poultry litter harbors a number of microorganisms belonging to aerobic and anaerobic bacteria species such as *Saprophytes*, *Enterococci*, coliforms, etc. (Bildirev, 1983; El-Jalil *et al.,* 2008; Barker *et al.,* 2010; Witkowska and Sowińska, 2017).

It is important to know the prevalence and distribution of different bacterial flora in poultry and its environment as many of them may be potential pathogen for poultry as well as humans that consume poultry products. In Ethiopia, the contribution of poultry farm environmental samples in harboring diarrheal causing bacterial pathogens specifically *Salmonella* and *E. coli* has not been studied. Particularly, there is very limited information available from eastern part of Ethiopia. Therefore, there is a need to conduct a study so as to fill the gap in relation to the distribution of *Salmonella* and *E. coli* among the environmental sources in poultry farms.

1.1. General Objective

The objective of this study was to isolate and identify *Salmonella* and *E. coli* from the environmental sources from poultry farms in Harar city and Haramaya town, Eastern Ethiopia.

1.2. Specific Objectives

The specific objectives of this study were:

- To estimate the prevalence of *Salmonella* and *E. coli* from litters, feeds, surface contacts, and water in poultry farms based on standard bacteriologic culture methods.
- To explore risks associated with the occurrence of *Salmonella* and *E. coli* in environmental samples of the poultry farms.

2. LITERATURE REVIEW

2.1. Diarrheal Causing Bacteria in Poultry Farm

The prominent diarrheal causing bacterial in the poultry farms include *Bacillus, Escherichia, Salmonella, Enterococcus, Campylobacter, Clostridium,* and *Lactobacillus* that have been shown to be of critical importance in tropical countries and elsewhere in the world (Vila *et al*., 2010; Chowdhuri *et al*., 2011; Hossain *et al*., 2011; Noohi *et al*., 2014; Mohanta *et al*., 2016). Avian *Salmonella* infections occur in poultry either acute or chronic form by one or more member of the genus *Salmonella*, under the family *Enterobacteriaceae* (Hofstad *et al*., 1984). *Escherichia coli* are a common pathogen for commercial poultry causing colibacillosis all over the world. It is a major cause of respiratory and septicemic diseases in broiler chicken causing mortality less than 5% and morbidity over 50% but in layer it affects the reproductive tract resulting failure of egg productivity and fertility (Barens and Gross, 1997). It may cause about 28% death in Sonali variety birds of Bangladesh (Biswas *et al*., 2006).

2.1.2. Phenotypic Characteristics of *Salmonella* and *E. coli*

Salmonella belongs to the family *Enterobacteriaceae*. The family is characterized by having wide range of bacteria which are Gram-negative rods up to 3µm in length, which ferment glucose and a wide range of other sugars and are oxidase-negative. They are catalase positive, non-spore-forming, facultative anaerobes which grow well on MacConkey agar because they are not inhibited by the bile salts in the medium. *Salmonella*e are usually motile and do not ferment lactose. The genus *Salmonella* contains more than 2,500 serotypes, based on a system devised by Kaufmann and White in which somatic (O) and flagellar (H) antigens are identified (Quinn *et al*., 2011). There are mainly two types of non-motile avian *Salmonella spp*. namely S. Gallinarum and S. Pullorum, which are responsible for fowl typhoid (FT) and pullorum disease (PD) of poultry, respectively. *S. Gallinarum* and *S. Pullorum* are short non-flagellated, non-spore forming, non-capsulated, gram-negative plump rods (Cheesbrough, 2006).

In general, the susceptibility of *S.* Pullorum and *S.* Gallinarum is about the same as that of members of the paratyphoid groups. They may survive for several years in a favorable environment, but they are less resistant than paratyphoid *Salmonella*e to heat, chemicals, and adverse environmental factors. For example, *S.* Gallinarum was killed within 10 minutes at 60°C; within a few minutes by direct exposure to sunlight; in 3 minutes by 1:1000 phenol, 1:20,000 dichloride of mercury, or1% potassium permanganate; and in 1 minute by 2% formalin (Snoeyenbos, 1991). It was also reported that *S.* Gallinarum retained viability up to 43 days when subjected to daily freezing and thawing (Orr and Moore, 1953). Organisms in liver survived more than 148 days at–20°C, even though they were accidentally thawed twice. *S. Gallinarum* can survive in feces from infected chickens up to 10.9 days when kept in a range house and 2 days less in the open (Smith, 1955).

Escherichia coli, which is member of the family *Enterobacteriaceae* is usually motile with peritrichous flagella and is often fimbriate. This lactose fermenter produces pink colonies on MacConkey agar and has characteristic biochemical reactions such as indole production, positive on methyl red test, negative for Voges-Proskauer and citrate utilization tests. Some strains produce colonies with a metallic sheen when grown on eosin–methylene blue agar. Haemolytic activity on blood agar is a characteristic of certain strains of *E. coli* (Quinn *et al*., 2011).

Escherichia coli possess no unique resistance capabilities and have a susceptibility pattern to chemical and physical agents typical of vegetative, Gram-negative bacteria. Inactivation of most strains will occur at temperatures ranging from 60°C for 30 minutes to 70°C for 2 minutes. Thorough pre-cleaning and/or presence of a germicide enhance thermal inactivation. The organism survives freezing and persists for extended periods at cold temperatures. Thermal inactivation in litter to achieve a 90% reduction in the number of bacteria is dependent on time and temperature ranging from 1–2 days at 37°C to 6–22 weeks at 4°C. Inactivation in litter is slower in the presence of high moisture and more rapid when free ammonia is present (Himathongkham *et al*., 2000).

Reproduction of most strains is inhibited by a pH of less than 4.5 or greater than 9, but the organism is not killed. Some virulent strains, *e.g.*, O157:H7, are acid tolerant, which permits

them to pass through the stomach without being killed. Organic acids are more effective than inorganic acids at inhibiting growth. Treatment with citric, tartaric, or salicylic acids significantly reduces coliform counts in poultry litter (240). A salt concentration of 8.5% prevents growth but does not inactivate the organism (Bell and Kyriakides, 1998).

2.2. Salmonellosis in Poultry

2.2.1. Etiology and the Diseases

The genus *Salmonella* are considered to have a two species named *Salmonella* enteric and *Salmonella* bongori. Serotyping differentiates the strains and they are referred as to by, for example *S. enterica* serotype Typhimurium or as S. Typhimurium (Quinn *et al*., 2011). Based on epidemiological, *Salmonella* can be placed into three groups; the first are those that infect humans only. This includes, S. Typhi, S. Paratyphi A, S. Paratyphi C. this group includes the agents of typhoid and paratyphoid fevers, which are the most severe of disease caused by *Salmonella*. The second was the host-adapted serovars (some of which are human pathogens and may be contracted from food), included are S. Gallinarum (poultry), S. Dublin (cattle), S. Abortusequi (equine), S. Abortusovis (sheep) and S. Choleraesuis (swine). The third is unadapted serovars (no host preference). There are pathogenic for humans and other animals. The epidemiology of the *Salmonella* is complex, which often make animals control of the disease is difficult. Animals are the reservoir of food born disease of *Salmonella* (Acha and Szyfres, 2001; Quinn *et al*., 2002).

Salmonella is capable of producing septicemic disease in most domestic and wild birds all over the world. Mortality in chickens has been reported 0 to 100% by PD and 10 to 93% by FT. Increased mortality, anorexia, sudden drop in egg production and white or yellow diarrhea are the characteristic clinical signs of the diseases. The gross lesions in chicks are unabsorbed yolk sac and turbid yellow colour fluids in the peritoneal cavity and in adult peritonitis, discrete, small, white necrotic foci in the liver and enteritis (Jordan *et al*., 2002; Saif, 2008).

Paratyphoid is a name given to infections of poultry by non-host-adapted *Salmonella* such as *Salmonella enteritidis* and *Salmonella typhimurium*. These infections are often subclinical in

laying birds (Cooper, 1994). *Salmonella pullorum, Salmonella gallinarum* and *Salmonella enteritidis* can infect the ovaries of hens and be transmitted through eggs. Pullorum disease or bacillary white diarrhea (*Salmonella pullorum*) infects young chicks and turkey poultry's up to 2 to 3 weeks of age. The mortality rate is high and affected birds huddle under a heat source and are anorexic, depressed and have whitish faecal pasting around their vents. Fowl typhoid (*Salmonella gallinarum*) can produce lesions in young chicks and poultry's similar to those of Pullorum disease. Characteristic findings include an enlarged, friable, bile-stained liver and enlarged spleen. As *Salmonella Pullorum* and *Salmonella Gallinarum* possess similar somatic antigens both have been eradicated from many countries by a serological testing and slaughter policy for Pullorum disease (Jordan *et al*., 2002; Saif, 2008).

2.2.2. Pathogenesis

Salmonella enters into host by ingestion, mediate acid resistance, few survive the stomach and move into the small intestine. Normal flora protects against colonization of administration of oral antibiotics facilitates establishment of infection. Entry of *Salmonella* usually occurs without mucosal damage in systemic infections, but enteric infection is characterized by local damage without septicaemia *Salmonella* infection with M cells in payer's patches is facilitated by fimbria adhesions. This is followed by ruffling of the target cell membrane which result in internalization of the bacteria in membrane bound vacuoles (Quinn *et al*., 2002; Bryan *et al.*, 1971). The ruffles facilitates uptake of the bacteria in membrane bound vacuoles or vesicles which often coalesce. The organisms replicate in these vesicles and are eventually released from the cells, which sustains only mild or transient damage. The complex invasion process is mediated by the product of a number of chromosomal genes, whereas growth within host a cell depends on the presence of virulence plasmids (Walderhaug (2007)). The incubation period of the *Salmonella* is 12-36 hours (Bean and Griffins, 1990).

2.2.3. Detection of *Salmonella*

Pathogen of *Salmonella* can be detected by isolate agent from stool sample, feed samples in cases of poultry. In general, for culture and isolation, the use of selective enrichment agar media is used. Broth for enrichment before sub-culture to these sugar agars after 24 hours is a usual procedure. Selenite enrichment broth or tetrathionate broth can be used to isolate highly

selective for *Salmonella*, especially *S. enterica serovar Typhi*. Agar and plates are incubated at 37°C overnight and growth identified by biochemical tests and slide agglutination tests (Bryan *et al.,* 1971). A number of commonly used media, including brilliant green (BG) agar and xylose–lysine– deoxycholate (XLD) agar, are used to differentiate *Salmonella* from other enteropathogens. On BG agar, *Salmonella* colonies and the surrounding medium show a red alkaline reaction. On XLD medium the colonies of most *Salmonella* serotypes are red (alkaline reaction) with black centres due to hydrogen sulphide (H2S) production (Quinn *et al*., 2011).

The main biochemical tests for most *Salmonella* species are the production of H2S, but not indole, in peptone water after incubation, together with certain sugar reactions. These latter comprise fermentation of glucose, mannitol, maltose and dulcitol and failure to ferment lactose, sucrose and salicin. These biochemical reactions can be conveniently carried out in the composite media now available for bacterial identification such as Kohn's or triple sugar iron (TSI) agar. The biochemical tests for identification of *Salmonella*s can be combined to form an identification test (Quinn et al., 2011; Saif, 2008). A number of these, such as the API 20E or Enterotube II systems, are currently available for purchase. Cultures that give the biochemical reactions characteristic of *Salmonella* species are then tested with the appropriate O and H sera until the group and serovar are identified.

Serological tests such as ELISA and agglutination techniques are of greatest value when used on a herd or flock basis. A rising antibody titre using paired serum samples is indicative of active infection. Molecular techniques have been developed for the identification of some serovars. For example, O'Regan *et al*. (2008) developed a multiplex PCR for the identification and differentiation of serovars Enteritidis, Gallinarum, Typhimurium, Kentucky and Dublin in poultry samples.

2.3. *Escherichia coli* Infection in Poultry

2.3.1. Symptoms of *E. coli* Infection

The incubation period of *E. coli* infection in chickens is 72-120 hours. The clinical sign initially may be diarrheal with abdominal cramps, which may turn into grossly bloody diarrheal in a few days. There is however, no fever. The symptoms of *E. coli* septicaemia are mainly referable to bacteraemia, end toxaemia and the effect of bacteria localization in a variety of tissue spaces throughout the body (Bryan, 1994; Jordan *et al*., 2002; Saif, 2008). *E. coli* infections cause many clinical manifestations such as airsacculitis, pericarditis, septicemia, and death of the birds (Hofstad *et al.,* 1984).

2.3.2. Pathogenesis

Escherichia coli are always found in the digestive tracts of poultry and in particularly large numbers in the lower part of the small intestine and caecum. The serovars most frequently causing colisepticaemia are also likely to be found in the throat and upper trachea following inhalation of dust containing *E. coli*. This pathogenic *E. coli* probably invade the bird's body from the respiratory tract following infection with other respiratory pathogens to produce the characteristic condition. Experimental infections are most easily established following infection with respiratory viruses such as coronavirus or rhinotracheitis virus. However, *E. coli* may also act as a primary pathogen when the bird's resistance is lowered by environmental stress and poor air quality (especially dust or high ammonia levels). Clinical colisepticaemia can be produced experimentally by parenteral injection or intratracheal administration of these pathogenic *E. coli* serovars into pathogen-free chickens, probably through avoiding respiratory tract defence mechanisms. The organism may also infect skin wounds or lesions leading to significant subcutaneous infections (Jordan *et al*., 2002; Saif, 2008).

2.3.3. Detection of *E. coli*

In suspected cases of colisepticaemia, isolation of the organism in pure culture from the blood or from parenchymatous organs is considered confirmatory. When enterotoxigenic strains of

E. coli are suspected, the presence of either enterotoxins or fimbrial antigens can be confirmed by immunological methods or molecular techniques such as the polymerase chain reaction (Quinn *et al*., 2011). Enterotoxins in the small intestine can be detected, using methods employing monoclonal antibodies (Carroll *et al*., 1990). For expression of fimbrial antigens, isolates should be subcultured on Minca medium. Fimbrial antigens can be identified using ELISA or latex agglutination (Thorns *et al*., 1989). PCR techniques using primers specific for genes encoding heat-labile and heat-stable enterotoxins may be used to identify enterotoxigenic strains of *E. coli* (DebRoy and Maddox, 2001).

Regarding to the detection of pathogenic strains with zoonotic potential, laboratory diagnoses involve culturing the food on MacConkey's agar followed by identification using serotyping with specific antisera. SLTs can be detected by ELISA and gene coding for them can be detected by DNA hybridization techniques. Sorbitol MacConkey's agar is recommended for isolation of *E. coli* O157:H7 from food and faeces samples, where they do not ferment sorbitol. Various immunoassay techniques can be used to detect SLT in food and faecal matter or cultures. Isolation becomes difficult beyond 1 week after onset of symptom (Quinn *et al.,* 2002; Radostits *et al.,* 2007).

2.3.4. Importance of *E. coli* in Human Infections

Enterohemorrhagic *E. coli* and verocytotoxin producing *E. coli* are being recovered in humans and animals and they constitute major food borne illness. *E. coli* O157:H7 is an important of serotype and it seems to predominate in most areas. The strains producing Vero toxin are shiga-like toxin (SLT) which produces diarrhoea in humans and animals. In most cases cattle are represents the main reservoir of *E. coli*. *E. coli* O157:H7 is transient inhabitant of gastrointestinal tract of normally ruminant. Source of infection is contamination of food by human and animal faeces. The organism can persist in manure, water trough and other farm location. The association of *E. coli* O157:H7 with raw meat, under cooked ground beef and raw milk lead to investigation of the role of cattle as a reservoir of the pathogens (Buchanan *et al.,* 1997).

2.4. Survival of *Salmonella* and *Escherichia coli* in Environmental Samples

2.5.1. Survival of *Salmonella* and *E. coli* in Water and Soil

Salmonella is frequently isolated from water sources (Cherry *et al*., 1972), which serve as bacterial reservoirs and may aid transmission between hosts (Foltz, 1969). Like *E. coli*, *Salmonella* is constantly released into the environment from infected humans, farm animals, pets, and wildlife, implying that Detection of *Salmonella* correlates with proximity to the sewage discharge area (Baudart *et al*., 2000). *Salmonella* can survive for 10 to 15 days in a septic system (Parker and Mee, 1982), whereas *E. coli* has a negative growth rate in this environment (Gordon *et al*., 2002). Compared to other bacteria, *Salmonella* has high survival rates in aquatic environments (Chao *et al*., 1987). The presence of *Salmonella* in marine environments does not vary seasonally and is independent of water temperature (Alonso *et al*., 1992). Thus, compared to *E. coli*, *Salmonella* appears to withstand a wider variety of stresses associated with environmental fluctuations and may persist in water environments for some time.

Previous studies have shown that *Salmonella* can be widely disseminated in soil, as a result of water currents, underground springs, and rain runoff carrying contaminated material (Chao *et al*., 1987; Abdel-Monem and Dowidar, 1990). It was also reported that *E. coli* has a low survival rate in soil (i.e., an average half-life of 3 days) (Temple *et al*., 1980; Bogosian *et al*., 1996), while *Salmonella* can survive and multiply for at least 1 year in this ecosystem (Thomason *et al*., 1977; Davies and Wray, 1996). Generally, *Salmonella* survives longer than *E. coli* in many nonhost environments and it was reported to be more resistant to killing by biotic factors (microbial predators or competing organisms) than *E. coli* in drinking water sources (Wright, 1989; Mezrioui *et al*., 1995).

2.5.2. Occurrence in Poultry Environments

Reports have shown that *Salmonella* spp. have extended survival in livestock farm environments such as 16-196 days in soil; 35 to >186 days in water; and 20 to 250 days in manure. Similarly, *E. coli* 0157:H7 can survive for 2 to >300 days in soil; 35 to >300 days in water; and 50 to >300 days in manure (Rogers and Haines 2005; Bowman, 2009). Moreover,

a study has shown that *Salmonella* can persist for more than 1 year in a poultry house, despite disinfections, suggesting that tong-term contamination of farms appears to be a widespread phenomenon (Davies and Wray, 1995). Several studies have shown that the occurrence of *E. coli* and *Salmonella* in samples from the poultry environment varies across geographic areas as it is shown in table 1.

Table 1. Prevalence of *E. coli* and *Salmonella* from poultry farm environmental samples

Country	Sample source	Prevalence	Bacteria	References
Cameroon	Poultry litter	44.1%	*E. coli*	Moffo *et al*. (2021)
Indonesia	Rinse water	52.5%	*Salmonella* spp	Yulistiani *et al*. (2019)
		75.0%	*E. coli*	
USA	Litter	15.2	*Salmonella*	Michael *et al*. (2021)
	Soil	11.5%	*Salmonella*	
USA	Poultry compost	63%	*E. coli*	Shepherd *et al.* (2010)
		26%	*Salmonella*	
Bangladesh	Poultry feed	71.4%	*Salmonella*	Chowdhuri *et al*. (2011)
		57.1%	*E. coli*	
New Zealand	Farm crevices	60.0%	*Salmonella*	Castañeda-Gulla *et al*. (2020)
	Drinker	45.0%		
	Feed loader	50.0%		
	Feeder	35.0%		
Ethiopia	Feed	0.0%	*Salmonella*	Betelhem *et al*. (2020)
	Floor swab	3.4%		
Nigeria	Feed	22.2%	*Salmonella*	Okoli *et al*. (2006)
Tanzania	Feed	29.4%	*Salmonella*	Mdemu *et al*. (2016)
Morocco	Poultry manure	55.5%	*E. coli*	El-Jalil *et al*. (2008)
		0.0%	*Salmonella*	
Uganda	Feed	51.7%	*E. coli*	Kevin (2020)
		0.0%	*Salmonella*	
	Litter	70%	*E. coli*	
		1.7%	*Salmonella*	

In poultry industry, millions of tons of by product materials such as litter, manure, feathers, wasted feed, slaughter waste, and bird carcasses are generated each year (Moore *et al.,* 1995, Williams *et al.,* 1999). Poultry waste materials are used as fertilizer in croplands, because of their nutrient values. Zoonotic pathogens present in wastes can potentially contaminate crops, and increase the risk of foodborne illness.

Microbial contamination of poultry production is an important first step in determining how such contaminants especially *Salmonella* pass through the food chain (Jones *et al.*, 1982), because the transmission of *Salmonella spp* through the environment has been shown to be cyclic, and poultry feeds have historically been viewed as important links for contamination in poultry (Williams, 1981). Although little is known about the relative significance of different sources of contamination of poultry feeds, it may depend partially upon the contamination levels of individual feed ingredients used in mixing the feed (Ekwuagana, 2004).

The disposal of by-products, such as poultry litter, and carcasses is a serious issue because of risks associated with microbial pathogens, and controlling the pathogen risks requires identifying improved pre-treatment methods capable of inactivating pathogens. Contamination of poultry drinking water with *Salmonella* could be a serious concern due to the potential for long-term exposure of the flock to the pathogen, resulting in intestinal colonization (Poppe *et al.,* 1986; Stersky *et al.,*1986).

Poultry environment like soil and drinking water (Eja *et al.,* 2012), faeces, litters and wastes (Igbinosa and Khan *et al.,* 2014), live, moribund and dead chickens (Hossain *et al.,*2008), meat, carcass, viscera, eggs, and poultry by-products (Adeyanju and Ishola, 2014; Laban *et al.,* 2014) carry out microbes of public and veterinary health importance. Recently, poultry feeds have been implicated in several poultry diseases of bacterial and fungal origin, suggesting that such feeds can potentially act as carriers for human as well as animal pathogens (Musa *et al.,* 2014).

2.5. Control of *E. coli* and *Salmonella* in Poultry

The control of *Salmonella* and *E. coli* infections in poultry is mainly by controlling the poultry farm environment, which is crucial to keep the quality and safety of poultry production. Effective protection of the poultry environmental can be achieved through the application of thermal processing; good hygiene practices; and flock vaccination. The control of salmonellosis and *E. coli* infections in poultry is mainly based on reducing the risk of exposure to infection (Jordan *et al*., 2002; Saif, 2008).

One important consideration in the control of *Salmonella* and *E. coli* in the farm environment is the role of insect vectors and other vehicles in the spread of the pathogens. It was reported that birds and flies are important vectors for rapid, widespread dissemination of *Salmonella* in the environment (Davies and Wray, 1996). Infection of wild bird populations with *Salmonella*e has been correlated with proximity to farms and/or the incidence of human salmonellosis in the same area (Monaghan *et al*., 1985). Likewise, adult muscoid flies (including the common housefly) can carry *Salmonella*, particularly on poultry farms (infection rate, 13%). *Salmonella* survives in flies for up to 4 weeks, which is the life span of the flies. Thus, flies that come in contact with contaminated materials (i.e., manure, food, and water) are capable of transmitting *Salmonella* (Mian *et al*., 2002).

Safe food preparation practices such as through cooking and reheating of food and adequate refrigeration, prevention of cross contamination; cleaning and disinfection of food preparation surfaces; exclusion of pets and other animals from food handling areas are important in controlling food-borne zoonotic pathogens. Because, intensively-reared, food producing animal are more likely to acquire infection and are also major source of human infection (Jay, 2000).

3. MATERIALS AND METHODS

3.1. Description of Study Areas

Harar town is located between 9°N latitude and 42°E longitude at the distance of about 526 km east of Addis Ababa (Figure 1). The altitude of the town is 1850 above sea level and its mean annual rainfall and humidity is 596 mm and 60.3%, respectively .The town has mean annual maximum and minimum temperatures of 25 and 10oC, respectively. The total human population of the town was estimated at 125,000 with a growth rate of 2.6% (HRLFO, 2019).

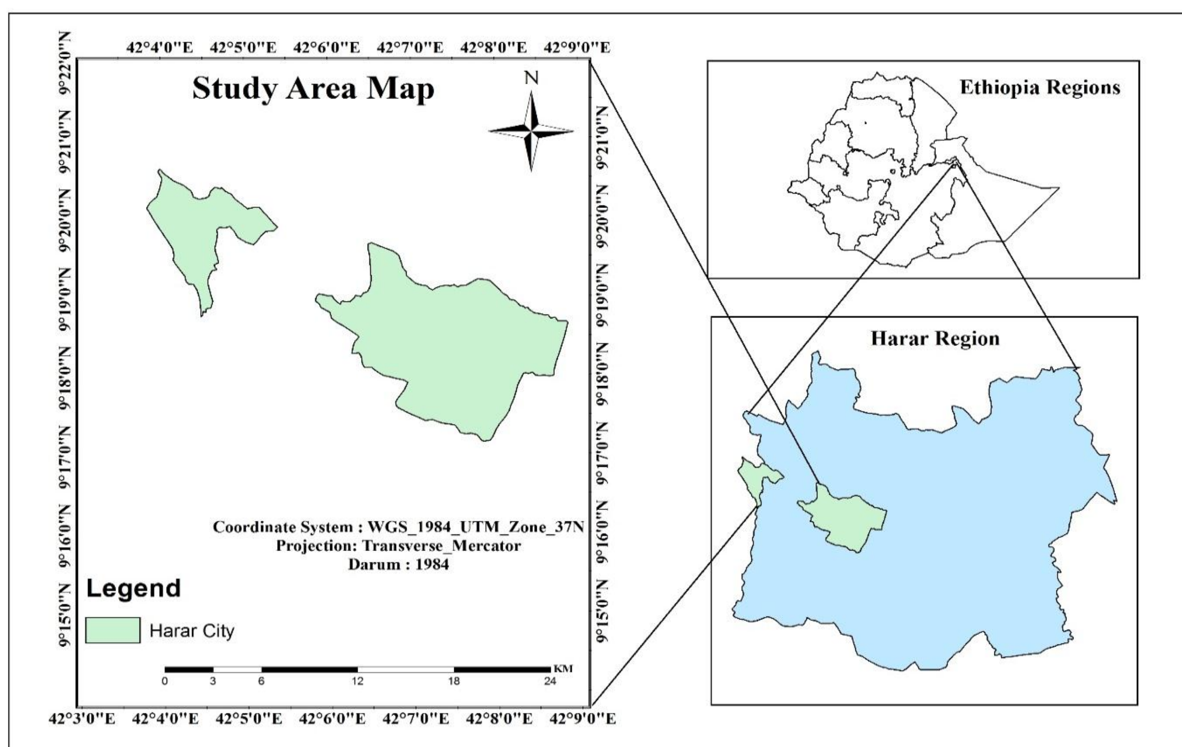

Figure 1. Map of Harar city.

Haramaya town is located in Oromia Regional State, which is 500 km far from Addis Ababa, the capital city of Ethiopia. Geographically, is situated at 410 51' 58" N latitude and 90024'10"S longitude (Figure 2). The area is located at 2000 m altitude above sea level and receives an average annual rain fall of approximately 900 mm, with a bimodal distribution pattern, peaking in mid-April and mid-August. There are four seasons, such as a short rainy

season (from mid-March to mid-May), a short dry season (from end May to June), a long wet season (July to mid-October) and a long dry season (end of October to February). Main pasture production is expected after the short rainy season, continuing until the end of the long wet season. Mixed type agriculture is the main occupation of the population of the area. Ecologically, the area has 65% midland and 35% lowland zones (Aman and Sitotaw, 2014). The two predominant soil types are 60% rigo soils and 40 % heavy black clay soil. The mean annual temperature ranges from 10°c to 18°C with a relative humidity of 65%. The husbandry system was sedentary system and the animals are reared mainly for marketing (HDADB, 2010).

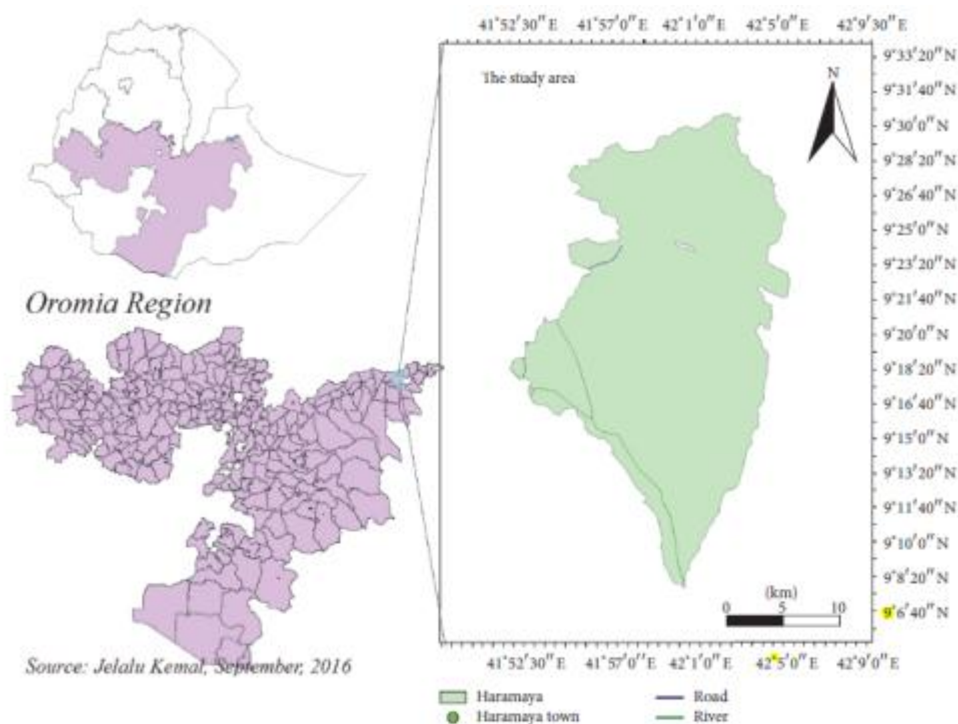

Figure 2. Map of Haramaya town.

3.2. Study Materials

The target material for this study were poultry farms in Harar city and Haramaya town.

3.3. Study Design

Across-sectional type of study was conducted from the October, 2020 to October, 2021 in the study area.

3.4. Study Farms Selection

The study was conducted on 13 poultry farms from 26 poultry farms by using purposive sampling that have been at least 800 chickens. The sampling strategy is resented in table 2.

Table 2. Sampling distribution between the study areas

No	Study Unit	Harar City	Haramaya Town	Total
1	Total poultry farms present	24	2	26
2	Total poultry farms selected	12	1	13

3.5. Sample and Data Collection

A total of 52 samples were collected randomly from 12 poultry farms from Harar (n= 48) and 1 Poultry Farm from Haramaya (n = 4), eastern Ethiopia. Sample collections include 13 litters, 13 water, and 13 feed and also 13 swabs from selected poultry farms. The collected samples were transferred on to sterile buffered peptone water in test tubes and transported to Haramaya University food microbiology and toxicology of central laboratory. Meanwhile, farm attributes and animal data such as flock sizes, breeds of poultry, source of feeds in the farms, source of water in the farms, frequency of cleaning house litters, use of disinfectants and foot baths, frequency of poultry manure disposal etc. for the individual farms were recorded in a structured record keeping format (Annex I).

3.6. Laboratory Methods

For isolation and identification of *E. coli* and *Salmonella* methods and procedures recommended by Quinn *et al*. (2002) was employed (Annex II). Firstly, the buffer Peptone water was used as primary enrichment media for both organisms *E. coli* and *Salmonella sp*.

Then selective media such as Mac-Conkey agar and EMB agars were used for *E. coli,* while XLD agars was used for *Salmonella sp* isolation.

For the isolation of *E. coli,* inoculated media were incubated at 37°C for 24hrs and observed for the typical colony character such as bright pink colonies on MacConkey agar and metallic green sheen on EMB agar. The suspected colonies were subculture on nutrient agar (24hrs; 37°C) and further subjected for primary identification such as gram's reaction, oxidation-fermentation (OF) test, oxidase test, and catalase reaction followed by further biochemical tests such as indole production, methyl red, Voges-Proskauer (VP), and citrate utilization test. Thus, *E. coli* was identified as gram-negative rod, fermentative, oxidase negative, catalase positive, positive pro indole production and methyl red test, but negative for VP and citrate tests.

For the isolation and identification of *Salmonella sp*, XLD agar plate were inoculated with peptone enriched samples and incubated at 37ºC for 24-48hrs. *Salmonella* was suspected if the colony appears as pink with a black center. Then, suspected colonies were transferred on to nutrient agar as it was done for *E. coli*. Primary identification was similar with *E. coli*. The biochemical tests conducted for the identification of *Salmonella* species were Triple Sugar Iron agar (TSI) sugar fermentation test, indole test, and citrate test, and urease test. Colonies producing an alkaline (red) slant with acid (yellow) butt with hydrogen sulphide production (blackening) on TSI, positive for citrate utilization (blue color), and negative for tryptophan utilization (indole test) (yellow-brown ring), and negative for urea utilization were considered as *Salmonella.*

3.7. Data Management and Analysis

All collected data were entered on Microsoft Excel Spreadsheet, coded, and filtered for errors. Descriptive statistics such as frequency and percentage were used to describe the occurrence of *E. coli* and *Salmonella* in different sample sources. Then risk factors associated with the occurrence of the isolated bacteria were computed with chi-square test statistical tool using SPSS version 20.0 (IBM) Software. The association of risk factors with the occurrence of the bacteria species was considered significant at p-value ≤ 0.05 (95% CI).

4. RESULTS

4.1. Prevalence of *Salmonella* in Environmental Samples of Poultry Farm

The current study showed that litter was found to harbour of *Salmonella* at higher proportion (84.6%), while the lowest was in water (38.4%) (Table 3).

Table 3. *Salmonella* occurrence on environmental sample sources of poultry farms.

Sample source	Number examined	Number positive for *Salmonella*	Prevalence
Water	13	5	38.46%
Feed	13	9	69.23%
Litter	13	11	84.6%
Swab	13	6	46.2%
Total	**52**	**31**	**59.61%**

4.2. Prevalence of *E. coli* in Environmental Samples of Poultry Farm

The current study showed that water was found to harbour *E. coli* at higher proportion (69.2%), while the lowest proportion was in swab (38.5%). In the feed and litter samples *E. coli* was isolated at equal frequencies (46.2%) (Table 4).

Table 4. *E. coli* occurrence on environmental sample sources of poultry farms.

Sample source	Number examined	Number positive for *E. coli*	Prevalence
Water	13	9	69.2%
Feed	13	6	46.2%
Litter	13	6	46.2%
Swab	13	5	38.5%
Total	**52**	**26**	**50%**

4.3. Risk Factors for *Salmonella* and *E. coli* Occurrences

4.3.1. Risk factors Associated with Occurrence of *Salmonella*

Among the variables examined, the hygiene status and manure disposal systems in the farms as well as the educational status of poultry house workers had significantly associated (P<0.05) with the occurrence *Salmonella* in the samples (Table 5). Meanwhile, the isolation frequency of the organism ranges from 0-100% from feed and water samples (Table 6).

Table 5: Risk factors associated within isolation of *Salmonella* based on chi-square test.

Risk factors		***Number examined***	***Positive for Salmonella***	***Prevalence***	***P-value***
Location	Haramaya	4	2	50%	0.142
	Harar	48	29	60.4%	
Farm size	Small	16	10	62.5%	0.519
	Medium	16	9	56.2%	
	Large	20	12	60%	
Educational level of poultry workers	Uneducated	13	9	69.2%	0.05
	Primary	15	11	73.3%	
	Secondary	12	6	50%	
	Higher education	12	5	41.7%	
Use of disinfectant	Yes	12	6	50%	0.512
	No	40	25	62.5%	
Poultry farm system	Industrialized	32	22	68.8%	0.056
	Tradition	20	9	45.9%	
Use of footbath	Yes	24	13	54.2%	
	No	28	18	64.3%	0.573

Clean	Yes	22	5	22.7%	0.000
	No	30	26	86.7%	
Manure disposal	Yes	22	5	22.7%	0.000
	No	30	26	86.7%	
Management of sick birds	Yes	12	5	41.7%	0.188
	No	40	26	65%	

Table 6: The distribution of *Salmonella* isolates based on sample source and risk factors

Risking Factors		**Environmental Source** *Number of examined, Positive for salmonella ,Prevalence*			
		Water	*feed*	*litter*	*swab*
Location	Haramaya	0(1)	1(1)	0(1)	0(1)
	Harar	5(12)	8(12)	11(12)	6(12)
Farm size	Small	2(4)	2(4)	4(4)	2(4)
	Medium	2(4)	2(4)	3(4)	2(4)
	Large	4 (5)	5(5)	4(5)	2(5)
Educational level of poultry workers	0=Un educa te	2(3)	3(3)	3(3)	1(4)
	1=Primary	2(4)	3(4)	4(4)	2(3)
	2=Secondar y	0(3)	2(3)	2(3)	2(3)
	3=Higher ed ucation	1(3)	1(3)	2(3)	1(3)
Use of disinfectant	Yes	3(3)	1(3)	2(3)	0(3)
	No	2(10)	8(10)	9(10)	6(10)
Poultry farm systems	Traditional	3(8)	1(3)	5(6)	2(3)
	Industrialize d	2(5)	8(10)	6(7)	4(10)
Use of footbath	Yes	4(6)	2(6)	5(6)	2(6)
	No	1(7)	7(7)	6(7)	4(7)
Clean	Yes	1(9)	0(2)	3(4)	1(7)
	No	4(4)	9(11)	8(9)	5(6)
Manure disposals	Yes	1(9)	0(2)	2(4)	1(7)
	No	4(4)	9(11)	8(9)	5(6)
Managements of sick birds	Yes	1(3)	1(3)	1(3)	0(3)
	No	8(10)	8(10)	9(10)	6(10)

4.3.2. Risk factors Associated with Isolation of *E. coli*

The occurrence of *E. coli* varied between the different variables examined in the present study. Among the the variables examined, farm size, use of disinfectant, poultry farm systems, use of footbath, and managements of sick birds tends to show variability in the frequency of *E. coli* detection (Table 7). However, only the farming system in the farms had significantly associated (P<0.05) with the overall occurrence *E. coli* in the samples. Moreover, the occurrence of *E. coli* based on sample source showed variations among associate risk factors. Meanwhile, the isolation frequency of the organism ranges from 0-100% from feed, litter, and swab samples, while the range for water sample was 20-100% (Table 8).

Table 7. Risk factors associated within isolation of *E. coli* based on chi-square test.

Risk factors		No examined	No. positive for *E. coli*	Prevalence	P-value
Location	Haramaya	4	2	50%	0.319
	Harar	48	24	50%	
Farm size	Small	16	9	56.2%	0.736
	Medium	16	7	43.8%	
	Large	20	10	50%	
Educational level of poultry workers	Un educate	13	6	46.2%	0.499
	Primary	15	8	53.3%	
	Secondary	12	7	58.3%	
	Higher education	12	5	41.7%	
Use of disinfectant	Yes	12	4	33.3	0.324
	No	40	22	55.5	
Poultry farm systems	Traditional	20	17	85.5	0.000
	Industrialized	32	9	28.1	
Use of footbath	Yes	24	11	45.8	0.781
	No	28	15	53.6	

Clean	Yes	22	11	50	0.222
	No	30	15	50	
Manure disposals	Yes	22	11	50	1
	No	30	15	50	
Managements of sick birds	Yes	12	4	33.3	0.324
	No	40	22	55.5	

Table 8. The distribution of *E. coli* isolates based on sample source and risk factors.

Risking Factors		***Positive for E. coli (Number of examined), Prevalence in:***			
		Water	Feed	Litter	Swab
Location	Haramaya	1(1),100%	1(1),100%	0(1),0.0%	0(1),0.0%
	Harar	8(12), 66.7%	5(12),41.7%	6(12),50%	5(12),41.7%
Farm size	Small	3(4), 75%	1(4), 25%	3(4),75%	2(4),50%
	Medium	2(4), 50%	2(4), 50%	1(4),25%	2(4),50%
	Large	4(5), 80%	3(5), 60%	2(5),40%	1(5),20%
Educational level of poultry workers	Un educate	3(3),100%	0(3),0%	2(3),66.7%	1(4),25%
	Primary	2(4),50%	3(4),75%	2(4),50%	1(3),33.3%
	Secondary	1(3),33.3%	3(3), 100%	1(3),33.3%	2(3),66.7%
	Higher education	3(3),100%	0(3),0%	1(3),33.3%	1(3),33.3%
Use of disinfectant	Yes	2(3),66.7%	1(3) ,33.3%	1(3),33.3%	0(3),66.7%
	No	7(10),70 %	5(10), 50%	5(10),50%	5(10) ,50%
Poultry farm systems	Traditional	8(8),100%	3(3) ,100%	4(6) ,66.7%	2(3),66.7%
	Industrialized	1(5),20%	3(10),30 %	2(7),28.7 %	3(10),30%
Use of footbath	Yes	4(6) ,66.7%	2(6) ,33.3%	3(6) ,50%	2(6) ,33.3%
	No	5(7), 71.5%	4(7) ,51.2%	3(7) ,42.9%	3(7) ,49.9%
Clean	Yes	6(9),66.7 %	2(2) ,100%	2(4) ,50%	1(7) ,14.3%
	No	3(4) ,75%	4(11), 36.4%	4(9),44.4 %	4(6) ,66.7%
Manure	Yes	6(9) ,66.7%	2(2) ,100%	2(4) ,50%	1(7) ,14.3%

disposal	No	3(4) ,75%	4(11), 36.4%	4(9) ,44.4%	4(6) ,66.7%
Managements	Yes	2(3) ,66.7%	1(3) ,33.3%	1(3) ,33.3%	0(3),0%
of sick birds	No	7(10),70 %	5(10), 50%	5(10), 50%	5(10), 50%

5. DISCUSSION

Enteric pathogens, especially *Salmonella* and *E. coli* are among the most important bacterial pathogens of domesticated chickens. The organisms are inhabitants of gastro-intestinal tract of poultry. However, various literatures and research works have shown that the farm environments are the main source of these enteric organisms in the causes of poultry disease in the intensive farming system. This is particularly important in the intensive poultry farms, since wastes are formed in the farms and if not properly managed, they could serve as a vehicle for the spread of high number of pathogens. Particularly, in farms where all operations are performed manually the source of contamination tends to be complex. Thus, the current study was conducted to elucidate the potential of poultry environments in harboring *Salmonella* and *E. coli.* The study was based on bacteriologic culture and identification of the bacteria using biochemical tests.

The current study was reported that the overall prevalence's of *Salmonella* from non-host samples in the studied farms was 59.61% and based on environmental sample source to be 84.6%, 69.23%, and 38.4%and 46.2% from litter, feed, swab, and water, respectively. Previous studies showed that *Salmonella* from poultry feeds was isolated at lower frequency. Thus, Betelhem *et al*. (2020) from Ethiopia reported 0.0% from poultry feed samples and 3.4% from poultry house floor swabs. Moreover, Okoli *et al*. (2006) reported 22.2% from Nigeria; Mdemu *et al*. (2016) reported 29.4% from Tanzania with a range from 21.1%-38.1% based on feed types formulated for layer, grower, starter and finisher. In USA, *Salmonella* occurrence in litter ranges from 6.1% to 26% (Shepherd *et al*., 2010; Michael *et al*., 2021).

In Canada, from litter samples reported *Salmonella* prevalence were: 7% by Alexander *et al*., (1968); 19%–89% by Higgins *et al*. (1982); 0%–2%, 30% and 0%–2% by Bhargava *et al*. (1983); 30% by Long *et al*. (1980); and 76% by Renwick *et al*. (1992). In Australia *Salmonella* prevalence's of 71% was reported from litter by Chinivasagam *et al*. (2010). In USA *Salmonella* prevalence's reported from litter ranges from 73% to 89% (Smyser and Snoeyenbos, 1969). From Nigeria, a far lower prevalence of *Salmonella* was isolated from environmental samples, in that only 2.67% of feed samples and 0% water samples harbors *Salmonella* along with other members of enteric bacteria such as *E. coli* and *Proteus mirabilisthis* (Madaki *et al*., 2019). The disparities of *Salmonella* prevalence could be due to differences in sampling, testing methods and difficulties in *Salmonella* isolation if the organism is found in low amount in the samples (El-Jalil *et al*., 2008; Kevin, 2020). Among risk factors examined, the hygiene status and manure disposal systems in the farms had significantly associated ($P<0.05$) with the occurrence *Salmonella* in the samples. This is due to the fact that *Salmonella* can multiply at high rate in the presence of organic loads as it is happened in the host environments (Thomason *et al*., 1977; Davies and Wray, 1996).

The overall prevalence of the *E. coli* from non-host samples in poultry farms was 50%. With regard to *E. coli*, the current study has shown that higher occurrence was observed in water samples (69.2%) followed by feed and litter at equal frequencies (46.2%), while the least was swab samples at 38.5%. This highest occurrence in water might be due to its suitable condition for survival and multiplication of numerous microbes. This finding is similar to a report from Morocco as reported by El-Jalil *et al*. (2008) with percentage of 55.5% from poultry manure and from USA by Shepherd *et al*. (2010), who reported that 63% of poultry compost harbor *E. coli*. In Australia, 100% litter samples harbors *E. coli* as reported by Chinivasagam *et al*. (2010). The lower frequency of *E. coli* from swab samples in the present study suggests that its survival is influenced by the specific niche, in which the organism is located. Previous studies have shown that despite the frequent contamination of environment due to fecal shedding, the organism has a negative growth with limited survival if in dry environment (Gordon *et al*., 2002). It was also reported that *E. coli* has a low survival rate in soil (i.e., an average half-life of 3 days) (Temple *et al*., 1980; Bogosian *et al*., 1996).

The prevalence of *E. coli* recorded (50%) is similar to a related study carried out in Bangladesh (57%) by Sultana *et al*. (2017). In Iraq it was reported to be 16% by Al-Musawi *et al*., (2016) and in Nigeria it was reported as 10.6% and 11% (Matthew *et al*., 2017, Okogun *et al*., 2016). The presence finding on high frequency of water contamination by enteric bacterial could be an indication of lack of proper water treatment in the farms. Among risk factors were examined, poultry farming systems had significantly associated (P<0.05) with the occurrence *E. coli* in the samples. This is due to the fact that in the traditional poultry management system, the operational units are not well-demarcated and there is high rate of cross-contamination between the different units, such as feeding area, feed handling/storage area, and production areas.

Moreover, the present study has shown that with the exception of water sample, the occurrence of *Salmonella* on different samples is higher than *E. coli*. This could be due to the fact that *Salmonella* generally survives longer than *E. coli* in many nonhost environments and it was reported to be more resistant to killing by biotic factors (microbial predators or competing organisms) than *E. coli* in drinking water sources (Wright, 1989; Mezrioui *et al*., 1995).

6. CONCULISIONS AND RECOMMENDATION

The present study indicated an overall prevalence's of 59.61% and 50% for *Salmonella* and *E. coli,* respectively from different environmental samples of poultry farms located in Harar and Haramaya town of eastern Ethiopia. The study revealed that the hygiene status, manure disposal systems, and farming systems were significantly associated with the occurrence of the isolated bacteria species. Moreover, it was found that litter and feed were the main harboring site for *Salmonella*, while water was found to be the main habitat for *E. coli*. In addition, the study has revealed that contact surfaces in the poultry farms are the potential sources for the organisms. As the study is based on cross-sectional design, it is difficult to estimate the survival rates of the bacteria species in different niches of the poultry farms. Generally, it is evident that large proportions of farm environments are contaminated with the tested bacterial species and possibly the environment are serving as rich source of nutrient for microbial growth and further spread to the poultry managed in the farms. In addition, the isolated bacteria could have a significant public health consequence due to direct transmission from the environment as well as infected poultry and their products through the food chain.

Therefore, based on above conclusion the following recommendations are forwarded.

- Farm owners should maintain the overall hygiene status and manure disposal systems.
- Poultry farms operational units should be based on modernized systems and in a way that is suitable for cleaning and disinfecting.

- In the control of infectious diseases, it is important to give high priority in controlling dissemination of bacterial pathogens in the environments.
- A detailed study is needed to rule-out the survival rate of bacterial pathogens in different settings of a poultry environment.
- A study based on molecular epidemiological tools is needed to elucidate the potential of different sources in the spread of the bacteria species to poultry.

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8. ANNEXXES

Annex 1: Questionary survey data collection formats

A. poultry farms owner's information

1. Kebele______________________________ Tel______________________________
2. Poultry farms Name: ______________________________
3. Litter change when new flock introduced to the house (a) No (b) Yes
4. House cleaning (a) use glove (b) bare hand cleaning
5. Poultry-house disinfection before placement (a) No (b) Yes
6. Boots for workers (a) No (b) Yes
7. Common use of working and protective materials between houses
8. Always boots dipping before entering poultry houses (a) No (b) Yes
9. Source of water for poultry (a) Well (b) Pipe
10. Storage of manure inside the farm compound
11. Poultry management types (a) industeralized (b) traditional
12. Washing of hands before handling poultry (a) No b) Yes
13. Regular cleaning of watering and feeding trough (a) No b) Yes
14. Breed of poultry: (a) local (b) exotic (c) cross
15. Flock size (number of birds in the farm) (a)<500 (b)500–1,000 (c)>1,000
16. Level of education of the farmer (a)Primary school (b)Secondary Certificate (c)University
17. Source of water in the farms (a)Rain water (b)Well water (c)Tap/Borehole
18. poultry manure disposal (a)Disposed weekly (b)Disposed monthly (c)Never
19. Management of sick birds in the farms (a)Isolated from healthy birds (b)No isolation
20. Use of disinfectants and footbaths (a)Yes (b)No

Annex 2: The list names of materials and laboratory procedures followed

The materials used in the laboratory was Autoclave and petri dishes masks and hand gloves incubator and refrigerator microscopy within their slides weighing balance, hot air oven and stovewater bath calibrated cylinder vortex machine bunsen burner wire loops alumunieum foil test tubes with racks 9 ml and 10 ml graduated cylinders1000 ml and 500 ml flasks 500 ml and 250 ml.

B. The list names of media used in laboratory for cultured and identification

For cultured Buffer peptone water, MacConkey agar and Ethylene Methyl Blue and XLD. For identification or biochemical tests NA, TSI and MR-VP medium, Urea broth and Basal media and Simon citrate agar. The reagent used for an identification was Methyl red, oil immersion and kovac' reagent alchol and safranine, iodine and glycirine crystal violets and potassium hydroxide (KOH) hydrogen peroxide (H2O2).

C. Laboratory procedures

<u>Procedure for gram staining</u>

After i was prepared all necessary reagents as well as materials, firstly i was applied a thin smear of the pure colonies from the growth media nutrient agar (NA). Then i was fixed them on the slide by passed the slide three or four times though the flame of a bunsen burner so that the colonies does not wash off during the staining procedure followed, then i was placed them smeared on a staining rack and overlaid the surface with crystal violet solution for 60 seconds. Then after 2 minutes of applied to the crystal violet stain pour off and i washed the remaining stain with an iodine, leaving the slide covered with for 15 seconds. Then the cropped off the iodine solution for 60 seconds and washed in alcohol unless no crystal violet dye is washed off anymore. Then the counter-stain with carbol fuchsine/ safranin for 60 seconds was added during the followed the procedures. Finally i was washed with water, then placed the smear in an upright position in a staining rack and then allowed the excess water to drain off and the smear and to dry.

I was interpreted as the following:

The gram positive bacteria was appeared in the forms blue, in this test i was focused only on the gram negative bacteria's specifically on *salmonella* and *E.coli*. So my focus is only on gram negative bacteria specifically *salmonella* and *E.coli* the presence of small red coloured rods confirms for *E.coli* and also for *salmonella*. Gram negative bacteria was stained red after i was followed the procedures for stain.

A. <u>Procedure for Catalase test</u>

Firstly i was taken the pure colonies i was characterized from NA to on the cleans microscopic slide then i was placed the drop of the 3% H2O2 on a glass slide. Then touched a sterile loop to a culture of the organism to be tested and pick up a visible mass of cells (colony). Then i was mixed the colonies within H2O2 on the cleans of the microscopic slides. Finally i was observed for immediate and vigorous bubbled on the cleans microscopic slide.

Interpretation: Bubbling was indicated as a positive test

: And no bubbling was indicated as a negative test.

B. <u>Procedures for Oxidase test</u>

Firstly as a catalase test procedures was followed pure colonies was taken from the NA to the cleans microscopic slides then i was applied the oxidase reagents 5% was dropped on the colonies on the slides within the oxidase reagent, finally i was read from their reactions after few a minutes, then i was interpreted the results as the followed for *E.coli* and *Salmonella* then i was observed for changing of the colonies in to blue colored for both organisms.

I was interpreted as the following:

A positive result was showed: Blue color appeared

A negative result was showed: No colour changed

C. <u>Procedure for Triple Sugar Iron agar(TSI)</u>

Firstly for TSI i was taken the pure colonies from NA for both organisms *Salmonella* and *E.coli* by used of straight inoculation needle, after incinerated by bunsen burner then i was started to taken of the colonies between this i was sterilized then i was inoculated on to the slant form prepared tsi, through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant. Finally after i was inoculated all of them by labeling on the tubes for different environmental samples for both organisms *Salmonella* and *E.coli* then after i was incubated them at 37 0c in the incubator for 18 to 24 hours. Then i was read the result as the followed.

I was interpreted: The positive result**:** For *E.coli* organism was showed on the slanted yellow slant, yellow butt, presence of gas bubbles, and absence of black precipitate in the butt was observed.

: The positive result**:** for *Salmonella* organism was showed on the slanted the colonies producing an alkaline (red) slant with acid (yellow) butt with hydrogen sulphide production (blackening) on TSI was observed.

D. **<u>Procedure for Sugar Fermentation Test</u>**

Firstly i was taken the colonies from NA to the basal mediums prepared after followed of autoclaved for 15 minutes then i was poured this medium on to the test tubes, then i was taken three different types of sugars for the seek of oxidation and fermentations. Then two tubes of the 0-F medium are heated in a beaker of boiling water immediately before use in order to drive off any dissolved oxygen. Then the each tube was inoculated with the unknown organism using a straight needle, stabbing the medium 3 to 4 times half way to the bottom of the tube. One tube of each pair is covered with 1 cm layer of sterile mineral oil or melted paraffin, leaving the other tube open to the air. Incubate both tubes at 35°c and examine for several day:

I was interpreted as the following:

Besides carbohydrate utilization, gas production and motility may also be confirmed with O-F medium.

	Open tube	Sealed tube
Unreactive	Green	Green
Oxidation	Yellow	Green
Fermentation	Yellow	Yellow

E. Methyl Red/Voges-Proskauer (MR-VP) medium test for

1. Procedure for MR (Methyl Red) test:

Firstly i was prepared the MR-VP medium followed by the manufacturer, then autoclaved for 15 minutes then i was poured in to the test tubes of 9 ml, then i was inoculated the colonies growth on NA, then incubated it in to an incubator at 37 0c for 24-72 hours, after incubated then i was applied in test tubes mixed within the colonies and MR-VP medium for MR 1.6 drops of methyl red was applied then i was seen the changing of the colour after incubated again in the incubator at 37 oc for 24-48 hours then i depicted theirs reaction by seen of formation of bright red colour holder as a positive results as i read the instruction of this test.

I was interpreted this test: The positive result: Bright red color was confirmed.

The negative result: Yellow color was confirmed.

2. Procedure for VP (Voges-Proskauer) test

For this test the same procedure was followed as MR test or because of the same media was prepared then i was poured from 10 ml MR –VP medium after inoculated the colonies growth on na then half for mr and as well as for VP 5ml then the different reagent was applied, so for this test procedures after i was prepared the reagent from the KOH and peptone water mixed thoroughly then applied on the half test or 5 ml of VP then again incubated at 37 oc for 24-48 hours the i was depicted their results based on the test procedure .

I was interpreted this test: The positive result**:** Red color showed

: The negative result: Yellow color showed

F. Procedure for Indole production

Tryptone water was prepared based on their manufacturer then colonies growth on NA was inoculated in to the test tubes filled by peptone water then incubated at 37 oc for 48 hours then removed from the incubator the kovac's reagent was applied on the test tubes filled with peptone water within the inoculated colonies after incubated was 0.5 ml of kovac's reagent added then i was confirmed their reaction to changing colors from pink colors to as the followed.

I was interpreted this test: The positive**:** Pink- to wine-colored ring was formed.

: The negative**:** No color was changed

G. Procedure for Simmon's Citrate Agar

Firsly i was prepared the Simmons Citrate Agar from the bottles based on the manufacturer, the after autoclaved for 15 minutes i was dispensed in to the test tubes in slant form, then the labeled colonies from NA are was inoculated in to the slant formed citrate, then i was labeled to each other's on the test tubes slanted followed for all my samples, then i was incubated them in the incubator at 37°c for 4 a days.

Then after i was depicted they reactions after incubated for an allowed days as the following. Only the organism capable of utilization a Simon Citrate as a source of carbon grow on the slanted and can produce a color was changed from green to blue.

I was interpreted this test: For *Escherichia coli-* Green color was appeared.

: For *Salmonella* - Blue color was appeared

H. <u>Procedure for urea broth (Urease test)</u>

Firstly i was prepared the Urea Broth based on the manfuter after autoclaved then i was putted the broth in the water bath then i was filtered the distilled water by filter paper then i mixed thoroughly the urease with the filtered distill water then i was added within the broth prepared in the water bath then i was inoculated the colonies growth on NA then mixed together Urea Broth and urease, finally after i inoculated the colonies incubated in the incubator for 24-48 hours then after, i was confirmed the reaction as the following.

I was interpreted from their reaction as the following:

The positive result was changed color formed yellow especially for *Salmonella.*

The negative for Urea hydrolysis (no colour change).