JIMMA UNIVERSITY

COLLEGE OF AGRICULTURE AND VETERINARY MEDICINE

SCHOOL OF VETERINARY MEDICINE

ISOLATION OF SALMONELLA FROM RAW CHICKEN EGGS, ANTIMICROBIAL SUSCEPTIBILITY AND ITS ASSOCIATED RISK FACTORS IN KONTA-AMEYA SOUTH WEST ETHIOPIA

MSc THESIS

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Isolation of *Salmonella* from Raw Chicken Eggs, Antimicrobial Susceptibility and Its Associated Risk Factors in Konta-Ameya Southwest Ethiopia

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A Thesis Submitted to Jimma University School of Veterinary Medicine, in Partial Fulfillment of the Requirements for the Degree of Masters in Veterinary Public Health (MVPH).

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We, the undersigned, member of the Board of Examiners of the final open defense by **Mesfin Mekuria** have read and evaluated his/her thesis entitled **"Isolation of Salmonella From Raw Chicken Eggs, Antimicrobial Susceptibility and Its Associated Risk Factors In Konta-Ameya South West Ethiopia"** and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree Master of Science in Veterinary Public Health

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DEDICATION

I dedicated this small piece of work to my families and my friends those who are always behind my success.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my own work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Jimma University, College of Agriculture 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. Brief quotations from this Thesis may be made without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this Thesis in whole or in part may be granted by the Head Department when in his or her judgment the proposed use of the material is in the interest of the scholarship. In all other instances, however, permission must be obtained from the author of the Thesis.

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BIOGRAPHICAL SKETCH

The author was born in Konta zone Ella Hanchano woreda, SWEPR State from his mother Aynate Anja and his father Mekuria Mechu on November 24, 1981. He attended his primary school at Womba Yemala Primary school, Junior Secondary School at Chida and Ameya his secondary school education at Jimma Comprehensive Secondary School. In 2006 he joined Alage ATVET College and graduated with diploma in animal health. Soon after graduation he was hired by the Konta Zone Livestock and Fisheries Office as an animal health assistant. In 2010 he joined in Haramaya University and graduated with a Bachelor degree in veterinary science (BVSc) in 2014. He again joined graduate program at Jimma University in 2022 to peruse his career in Veterinary Public Health (MVPH).

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LIST OF ACRONYMS AND ABBREVIATIONS

ACMSF	Advisory Committee on the Microbiological Safety of Food
AST	Antimicrobial Sensitivity Test
ATCC	American Type Culture Collection
BPW	Buffered Peptone Water
CAFO	Concentrated Animal Feeding Operations
CDC	Center for Disease Control
CFSAN	Center for Food Safety and Applied Nutrition
CFU	Colony Forming Unit
CIDRAP	Center for Infectious Disease Research and Policy
CLSI	Clinical and Laboratory Standards Institute
DSM	Diagnostic Services of Manitoba
EFSA	European Food Safety Authority
ERS-USDA	Economic Research Service United States Department of Agriculture
ESR	Environmental Science and Research Limited
FDA	Food and Drug Administration
FSA	Food Standards Agency
HPA	Health Protection Agency
ICMSF	International Committee on the Microbiological Specifications for Foods
ISO	International Standard Organization
KZADO	Kenta Zone Agricultural Department Office
KZFEDO	Konta zone Finances and economic development office
MAP	Modified atmosphere packaging
MAR	Multiple Antimicrobial Resistance
MKTT	Muller-Kauffmann Tetrathionate broth
NCCLS	National Committee for Clinical Laboratory Standards
RV	Rappaport Vassiliadis medium
TSI	Triple Sugar Iron
XLD	Zylose Lysine Deoxycholate agar

ABSTRACT

Salmonellosis is the major food-borne disease in the world with a serious public health problem. A cross-sectionals study was conducted from March 2023 to April, 2024 to determine the prevalence, antimicrobial susceptibility pattern and associated risk factors of *Salmonella* isolated from fresh raw chicken eggs collected at Konta-Ameya poultry farms and open local markets in southwest Ethiopia. The samples were examined by using culture technique and were confirmed by biochemical test. From the total of 384 eggs examined for *Salmonella*, 34(8.85%) were positive, of which 26 (6.77%) and 8 (2.08%) were isolated from eggshells and egg contents, respectively. There is no significantly difference between prevalence of *Salmonella* in eggs from the open market (11.46%) and poultry farm (6.25%). The prevalence of *Salmonella* in egg content of the open market (3.65%) was also significantly higher than the prevalence of *Salmonella* in egg contents from poultry farms (0.52%). The prevalence of *Salmonella* in eggs from different chicken breed sources was 11.59%, 8.15% and 4.71% for local, cross and exotic breeds respectively. This is presumably due to unequal exposure to the risk factors as the breeds were housed in different house system. All 34 isolates of *Salmonella* were tested for their antimicrobial susceptibility for ten different antimicrobials. Of these *Salmonella* isolated showed high susceptibility to Nalidixic acid (76.47), followed by Ceftriaxone (61.76%) and Gentamycin (58.82%). Higher resistance was observed for Ampicillin (67.65%), followed by Amoxicillin (61.76%) and Clindamycin (55.88%). All of the isolates were resistant to at least one antimicrobial agent. For associated risk factor determination, questionnaire survey was also carried out on 75 egg sellers, farmers and consumers (40 egg sellers and 35 farmers and consumers). Their preferred form of egg consumption revealed that 70.67% preferred cooked eggs while 29.33% showed a preference for raw eggs. Thirty-four (45.33%) respondents who practiced eating raw eggs faced problems like nausea and vomiting, abdominal discomfort and diarrhea, while 41(54.67%) faced no problems. The presence of drug-resistant *Salmonella* contamination and habit of raw egg consumption is of significant public health concern. Therefore, eggs should be handled, transported, stored, and cooked correctly to reduce the risk of contamination from the pathogen. Public health initiatives like better food hygiene and health education are crucial in general.

Keywords: *Salmonella, Prevalence, Antimicrobial Susceptibility, Risk factors, Raw eggs, Konta-Ameya*

1. INTRODUCTION

1.1. Back Ground of the Study

Poultry, egg and egg products are among the most nutritious foods on earth and they share an important part of the human diet. However, they are perishable just like meat, fish and other food items, and consumption of improperly handled poultry and egg products is closely associated with negative health impacts causing food-borne illnesses, such as salmonellosis (Shahzad *et al,* 2012). Poultry eggs, meat and their products are the common vehicles of *Salmonella* to humans (Abebe *et al*., 2020). *Salmonella* is a leading cause of food-borne illness in many countries with eggs and poultry being important vehicles of transmission. During the past two decades *S. Enteritis's* has become a leading serotype causing human infections, with hen eggs being a principal source of the pathogen (Shaji *et al.*, 2023).

Salmonella is a species in the genus with worldwide public health implications and is the major cause of food borne disease, accounting for the deaths of thousands of people worldwide (Lee *et al*., 2015). The prevalence of *Salmonella* in animals is a continuous threat to human health (Ame *et al.,* 2022). *Salmonella* is widely distributed in nature and cause a spectrum of diseases in man, animals and birds. A total of 16 million cases of inflammatory fever, 1.3 billion cases of gastroenteritis and 3 million deaths from *Salmonella* infections worldwide (Pui *et al.*, 2011).

The genus *Salmonella* is gram negative, motile, facultatively anaerobic organisms belonging to the family *Enterobacteriaceae* (Bula-Rudas *et al*., 2015). The genus *Salmonella* contains two species, *Salmonella enterica*, which consists of six subspecies, and *Salmonella bongori* the first comprises six subspecies designated by Roman numerals (Figure 1) (Agbaje *et al*., 2011). *Salmonella* species can be classified into typhoid and nontyphoid (NTS) due to their ability to develop specific pathologies in humans (Hendriksen *et al*., 2011). The genus includes a total of more than 2,600 different serotypes (Sun *et al*., 2020). They differ from each other by their flagellar (H), Virulent (Vi) and somatic (O) antigens structures (Yachison *et al*., 2017). *S. enteric* subspecies I (*enteric*) is the most isolated subspecies in animals and is found in 99% of human isolates.

Egg contents may be contaminated with *salmonellae* by two routes: vertical transmission (transovarian) or horizontal (trans-shell) transmission (Awny *et al.*, 2018). The transmission of bacteria from the parent to the infant is vertical transmission. In *Salmonella* infection in poultry which is caused by serovar *enteritis* with a particular preference for the chicken reproductive system, vertical transmission is of great concern. In this case, it happens by transovarial infection when the mother bird has a systemic infection that results in ovary infection and egg production in the oviduct. Bacteria migrate from the cloacae into reproductive organs (Dos-Santos *et al*., 2019). Horizontal transmission is usually derived from fecal contamination on the eggshell with penetration after the egg is laid. It also includes contamination through environmental vectors, such as farmers, pets and rodents. Many different serotypes of the genus *Salmonella* may be able to contaminate egg contents by migration through the eggshell and membranes. Such a route is facilitated by moist eggshells, storage at ambient temperature and shell damage (Cader *et al*., 2014).

Ethiopia has approximately 56.87 million chickens, the majority (95%) of which is kept in low-input low-output village chicken production systems (Duguma, 2017). *Salmonella* was found in large numbers and was widely distributed in Ethiopia. The number of *Salmonella* outbreaks in humans in the country has increased significantly in recent years (Mengistu *et al.*, 2020). Much more is now known about the scope of food borne illness and how severe it can be, both in terms of acute illness and long-term consequences. Various percentages of *Salmonella* isolates were found in Ethiopian towns, according to studies (Kemal *et al.*, 2015).

In many countries, *Salmonella* spp. is controlled in the egg production chain. In addition, storing eggs in a cool area (below 15°C) and keeping eggs separate from other foods is important to avoid possible *Salmonella* cross-contamination and keep eggs safe (Hans and Dean, 2006). In Ethiopia from the total 366 chicken eggs examined for *Salmonella* prevalence, 27 (7.4%) were positive, from which 23 (6.3%) and 4 (1.1%) were found from eggshells and egg contents, respectively (Hailu *et al.*, 2021). A total of 384 samples from various poultry farms in Bishoftu and Adama were collected, and 62 (16.15%) of the isolates tested positive for *salmonella* (Asefa and Duga, 2022).

Poultry production uses antimicrobial agents for the prevention and treatment of infectious diseases, as well as well as growth promotion. The high use of antibiotics can lead to increased antibiotic resistance. Antibiotics as prevention are used routinely in most cases. The combination preparation of amoxicillin and Celestine (60.8%) was the most widely used in several farms. Farmers do not even evidence of the purpose of using antibiotics. The high use of antibiotics without a prescription is due to the perception of farmers that the use of antibiotics has no side effects and is at a low cost as an effort to prevent disease (Niasono, *et al.,* 2019). Amongst *Salmonella* spp. antimicrobial resistance is a well-confirmed phenomenon and antimicrobial-resistant *Salmonella* is increasingly associated with the use of antimicrobial agents (Teferi, 2020).

Antibiotic-resistant *Salmonella* infections of both humans and animal are universal concerns, particularly in developing countries where the risk of infection is high because of unhygienic living conditions, close contact and sharing of houses between animals and humans (Saad, 2022) and the traditions of consumption of raw or undercooked animal-origin food items. Ultimately, increases in bacterial antimicrobial resistance pose a considerable threat to public health, especially for vulnerable populations including young children the elderly and immunocompromised individuals (Kemal *et al.*, 2015).

Concentrated animal feeding operations (CAFOs) in agricultural practices have evolved to accommodate food consumption rates with increased agricultural output at the risk of introducing antimicrobial resistant pathogens into the environment. In addition, several studies have suggested that characteristics of agricultural environmental settings, including animal crowding, CAFO hygiene, temperature, ventilation control and stress, can influence antimicrobial resistance and pathogen risk (Kemal *et al*., 2015).

1.2. Statement of the Problem

Salmonella is a species in the genus with worldwide public health implications and is the major cause of food borne disease, accounting for the deaths of thousands of people worldwide (Lee *et al*., 2015). The prevalence of *Salmonella* in animals is a continuous threat to human health (Ame *et al.,* 2022). In Ethiopia as in other developing countries, it is difficult to evaluate the burden of salmonellosis because of the limited scope of studies and lack of coordinated epidemiological surveillance systems. In addition, under-reporting of cases and

the presence of other diseases considered to be of high priority may have overshadowed the problem of salmonellosis (Kebede *et al*., 2016).

Salmonellosis is endemic in the country and there is a desire to strengthen the monitoring and surveillance of salmonellosis using suitable diagnostic tools so as to prevent and control its occurrence. In addition to this, the extent of *Salmonella* contamination of eggs sold on local open markets and farms, and the antimicrobial profile of the *Salmonella* isolates has not been adequately studied and very limited information exists in Ethiopia and none in the Konta zone.

The presence of resistant organisms in poultry and poultry products for consumption is a safety concern to the population and a therapeutic concern for physicians which might pose prolonged treatment in cases of outbreaks, delayed recovery or treatment failure (Kemal *et al.*, 2016). Knowledge is scarce concerning poultry farm development associated with antimicrobi al resistance and food-borne bacteria. Information on the antimicrobial resistance patterns of the *Salmonella* isolates from chicken table eggs could be useful for successful treatment, as well as planning strategic use of drugs to minimize resistance in the future (Abreu *et al*., 2023)

Insufficient information at hand in this regard is the main factor that hindered efforts to prevent and control the disease. Based on the ubiquitous nature of *Salmonella* and previous studies made in Ethiopia, it was hypothesized that eggs sold in local markets and poultry farms may serve as a source of *Salmonella* species particularly among those raw egg consumers and a considerable proportion of them might have developed resistance to antimicrobials that are commonly used both in the veterinary and public health sectors. So having this understanding, this study is designed to fill the above gap in the study area concerning antimicrobial susceptibility patterns and public health importance of *salmonella*, response strategies and try to answer the following questions:

What is the extent of occurrence of *Salmonella* in raw chicken egg collected from open markets and poultry farms of Ameya district? What are the antimicrobial susceptibility patterns of *Salmonella* isolate from raw chicken eggs? What are the risk factors of *Salmonella* associated with raw eggs in the study area?

1.3. Objectives of the Study

1.3.1. General Objective of the Study

The main objectives of the present study to isolate *salmonella* from raw chicken eggs and to determine antimicrobial susceptibility pattern and associated risk factors in Konta-Ameya, southwest Ethiopia collected from the open markets and selected poultry farms.

1.3.2. Specific Objectives of the Study

- To determine the prevalence of *Salmonella* spp. in egg shells and egg contents of raw chicken eggs sold at local markets and selected poultry farms and assess associated risk factors.
- To determine the antimicrobial susceptibility patterns of the *Salmonella* isolates.

2. LITERATURE REVIEW

2.1. History of *Salmonella*

The genus *Salmonella* was named after Daniel E. Salmon who first reported the isolation of *Salmonella* from a pig in 1885 and named the organism Bacterium *Choleraesius* (currently known as *Salmonella*). A non-human *Salmonella* spp., *S. Choleraesius*, was isolated from a swine's intestine by Theobald Smith (1859-1934) under the direction of Daniel E. Salmon (1850-1914) in 1885 (Ellermeier and Slauch, 2006). Dr. Salmon was the administrator of the USDA research program, and thus the organism was named after him (Grossman, 2016; FDA/CFSAN, 2008).

It is conjectured that *Salmonella* and *E. coli*, which evolved from a common ancestor emerged about the time of the emergence of mammals, and emerges as mammalian and avian pathogens through the acquisition of pathogenicity islands and of a virulence plasmid plus through variation in lipopolysaccharide antigens (Kemal, *et al.,* 2015). It is hypothesized that accumulation of single mutations, insertions or deletions with the genome of modern-time *Salmonella* appears to have generated many pseudo genes, suggesting its recent evolutionary origin (Muktar *et al.,* 2015). *Salmonella* uses an array of virulence genes as part of its mechanism of pathogenesis (Ruimin *et al.*, 2019). These genes encode proteins that help the bacteria to evade the host's immune system, colonize and survive in host tissues, and cause inflammation and tissue damage (Jajere, 2019; Dos-Santos *et al.*, 2019). Many of these virulence genes are located on pathogenicity islands known as *Salmonella* pathogenicity islands (SPIs), which are thought to be acquired by horizontal gene transfer (Kombade *et al.*, 2021). Various *Salmonella* strains also contain plasmids, which have virulence and AMR genes (Bakkeren *et al*., 2019; Silva *et al*., 2019). *Salmonella* plasmids are usually small, circular pieces of DNA that are found in some strains of *Salmonella*. However, some strains also carry large *Salmonella* virulence plasmids (Silva *et al*., 2017; Naushad *et al*., 2020).

2.2. Characteristics of *Salmonella*

The family *Enterobacteriaceae* consists of Gram-negative, facultatively anaerobic non-spore-forming rods. *Salmonella* conforms to the general definition of the family (Perdomo, 2020). These organisms usually occur as short rods, measuring 2-4 micrometer in length and 0.5

micrometer in diameter. Occasionally, they develop into longer pleomorphic forms or very short coccobacilli after prolonged culture on laboratory media (Dugan, 2022). Members of the genus are motile by peritrichous flagellation except *S. Pullorum* and *S. Gallinarum*, which lack flagella. Non-motile variants can also arise as a result of a faulty assembly of flagellar subunits or deficiencies in the motor functions of these appendages. *Salmonellae* are chemoorganotrophic, with an ability to metabolize nutrients by the respiratory and fermentative pathways (Jwad, 2019).

2.2.1. Biochemical Characteristics

Salmonellae are generally unable to ferment lactose, sucrose or salicin, although glucose and certain other monosaccharaides are fermented with the production of gas. They are usually catalase positive, oxidase negative and reduce nitrates to nitrites. The microorganisms use citrate as the sole carbon source, decarboxylate lysine, arginine and ornithine and produce hydrogen sulphide. The methyl-red reaction is positive, the Voges-Proskauer test is negative and indole is negative. Phenylalanine is not delaminated, urea is not hydrolysed, gelatin is not liquefied rapidly in nutrient media and neither Dnase nor Lipases are produced. *Salmonellae* may harbor temperature phages or plasmids that code for metabolic characters used in identification (e.g. H_2S, lactose or sucrose fermentations) (Mabogo, 2004; Molbak *et al.*, 2006).

2.2.2. Growth and Destruction

Salmonellae are able to grow on a large number of culture media and produce visible colonies well within 24h at about 37^{o}C. The parameters of pH, water activity (aw), nutrient content and temperature are all interrelated for *salmonellae*, as they are for most other bacteria (Trinetta *et al*, 2019; D'Aoust, 2001). Most *Salmonella* serotypes grow at temperature range of 5 to 47°C with optimum temperature of 35 to 37°C but some can grow at temperature as low as 2 to 4°C or as high as 54°C. They are sensitive to heat and often killed at temperature of 70°C or above. *Salmonella* grow in a pH range of 4 to 9 with the optimum between 6.5 and 7.5. They require high water activity (aw) between 0.99 and 0.94 (pure water aw=1.0) yet can survive at water activity less than 0.2 such as in dried foods. Complete inhibition of growth occurs at temperatures less than 7°C, pH less than 3.8 or water activity less than 0.94 (Pui *et al.*, 2011).

Salmonellae are unable to tolerate high salt concentration Brine above 9% is reported to be bactericidal. Nitrite is effective, with the effect being greatest at the lower pH values. This suggests that the inhibitory effect of this compound is preferable to the undissociated HNO_2 molecule with respect to heat destruction all *salmonella* are readily destroyed at milk pasteurization temperatures. Some investigators also found that cells grown at 44°C were more heat resistant than those grown at either 15ºC or 35ºC (Christieans *et al*., 2018). Some food products are packaged under vacuum or gaseous mixtures of carbon dioxide, nitrogen and oxygen to increase product quality and extend shelf life. The benefits of modified atmosphere packaging stem are from the inhibition of endogenous spoilage micro flora and enhanced organoleptic stability of the product. The high concentrations of carbon dioxide (>50%v/v) commonly used in MAP inhibit the growth of *Salmonella* spp. but exert little or no effect on survival (Czerwiński *et al*., 2021).

2.3. Classification and Nomenclature of the Genus *Salmonella*

Historically *Salmonella* had been named based on the original places of isolation such as *S. London* and *S. Indiana*. This nomenclature system was replaced by the classification based on the susceptibility of isolates to different selected bacteriophages which is also known as phage typing. Phage typing is generally employed when the origin and characteristic of an outbreak must be determined by differentiating the isolates of the same serotype. It is very reproducible when international standard sets of typing phages are used more than 200 definitive phage types (DT) have been reported so far. For example, *S. Typhimurium* DT104 designates a particular phage type for *Typhimurium* isolates (WHO, 2004; Pui *et al*., 2011).

Epidemiologic classification of *Salmonella* is based on the host preferences. The first group includes host-restricted serotypes that infect only humans such as *S. Typhi*. The second group includes host-adapted serotypes which are associated with one host species but can cause disease in other hosts serotypes such as *S. Pullorum* in avian. The third group includes the remaining serotypes. Typically Enteritis's, *S. Typhimurium* and *S. Heidelberg* are the three most frequent serotypes recovered from humans each year (Afendy *et al*., 2022; D'Aoust, 2001; WHO, 2004).

The genus consists of two species: the first is *S. enterica* which is divided into six subspecies (Table1): *S. enteric* subsp. *enterica, S. enteric* subsp. *salamae*, *S. enteric* subsp. *Arizonae*, *S. enteric* subsp. *diarizonae, S. enteric* subsp. *Houtenae* and *S. enteric s*ubsp. *Indicia*. The second group is *S. bongori* (formerly called *S. enteric* subsp. *bongo*) (Park *et al*., 2020; Jacobsen *et al*., 2013). *S. bongori* are mainly isolated from cold-blooded animals and account for less than 1% of clinical isolates while *Salmonella enterica* sub species *enterica* is mainly isolated from warm-blooded animals and accounts for more than 99% of clinical isolates. Under the modern nomenclature system, the subspecies information is often omitted and culture is called *S. enteric* serotype *Typhimurium* and in subsequent appearance, it is written as *S. Typhimurium*. This system of nomenclature is used nowadays to bring uniformity in reporting (Bhunia and Bhunia, 2018). Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar (H) antigens, somatic (O) antigens and virulence (Vi) capsular K antigens. This was adopted by the International Association of Microbiologists in 1934. Agglutination by antibodies specific for the various O antigens is employed to group *Salmonella*e into the 6 sero groups: A, B, C_1, C_2, D and E. For instance, *S. Paratyphoid* A, B, C and *S. Typhi* express O antigens of sero groups A, B, C_1 and D, respectively. More than 99% of *Salmonella* strains causing human infections belong to *Salmonella enterica*. Although not common, cross-reactivity between O antigens of *Salmonella* and other genera of *Enterobacteriaceae* do occur (Pui *et al*., 2011; Teklemariam *et al*., 2011).

Therefore, further classification of serotypes is based on the antigenicity of the flagellar H antigens which are highly specific for *Salmonella* (Pui *et al*., 2011). In brief, O antigens are lipopolysaccharide (LPS) of the outer bacterial membrane. They are heat stable, resistant to alcohol and dilute acids. H antigens are heat-labile proteins associated with the peritrichous flagella and can be expressed in one of two phases. The phase 1H antigens are specific and associated with the immunological identity of the particular serovars whereas phase 2 antigens are non-specific antigens containing different antigenic subunit proteins which can be shared by many serovars. K antigens which are heat- sensitive carbohydrates are produced by *Salmonella* serovars that express a surface-bound polysaccharide capsular antigen (Oludairo, *et al*., 2011).

Table 1. *Salmonella* species, subspecies, serotypes and their usual habitats

***Salmonella* species and subspecies**	**Number of sero-vars with in subsps.**	**Usual habitat**
S. enteric	2594	
S. enteric subsp. *Enterica*	1586	Warm blooded animal
S. enteric subsp. *Salamae*	522	Cold blooded animals and environment
S. enteric subsp. *Arizonae*	102	Cold blooded animals and environment
S. enteric subsp. *Diarizonae*	308	Cold blooded animals and environment
S. enteric subsp. *Houtenae*	76	Cold blooded animals and environment
S. enteric subsp. *Indica*	13	Cold blooded animals and environment
S. bongori	22	Cold blooded animals and environment
Total	2,607	

Source: (CDC, 2016; Ryan *et al*., 2017)

Table 2. *Salmonella* nomenclature in use at CDC, 2008

Taxonomic position	**Nomenclature**
Genus (italics)	*Salmonella*
Species (italics)	• *enterica*, which includes subspecies I, II, IIIa, IIIb, IV and VI • *bongori* (formerly subspecies V)
Serotype (capitalized, not italicized)	• The first time a serotype is mentioned in the text; the name should be preceded by the word "serotype" or "ser". • Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II to IV, and VI and *S. bongori* • Members of subspecies II, IV and VI and *S. bongori* retain their names if named before 1966

Source: CDC, 2008

Table 3. Common *Salmonella*serovars that infect human and animal and syndrome they induce

Species	**Serotype**	**Common syndrome**
Cattle	*S. Dublin and S. Typhimurium*	Septicemia, acute and chronic enteritis and abortion
Horse	*S. Typhimurium*	Septicemia
Pig	*S. Choleraesuis, S. Typhimurium*	Septicemia, typhocolities and abortion
Chicken	*S. Typhimurium*	Septicemia, acute colitis and abortion
Chicken	*S. Pullorum, S. Gallinarium*	Septicemia, acute and chronic enterities
Human	*S. Enteritidis, S. Typhi, S. Paratyphi*	Gastroenteritis and bacteraemia

Source: (Patrick, *et al.*, 2007; Girmont and Weill, 2007)

2.4. Epidemiology

Salmonella is a major cause of bacterial food-borne diseases in both industrialized and developing countries, with varying incidences across these regions (Abebe *et al.,* 2020). The wide variations in the national prevalence of salmonellosis likely arise from limited scope of studies and lack of coordinated epidemiological surveillance systems, under-reporting of cases and the presence of other diseases considered to be of high priority (Gebre, 2023; Molla *et al.*, 2003).

The last few decades have witnessed marked increases in the incidence of human *Salmonella* infections. Several factors, including tourist travel, international movement of foods and food ingredients, animal feed trade and the importation of infected animal replacement stocks contribute to the national succession of *Salmonella* serotypes in human populations and in the food chain (Teklemariam *et al.,* 2023). The prominence of agricultural products as major reservoirs of *Salmonella* spp. arises from the ubiquity of the microorganism in the natural environment, the intense on-farm husbandry practices that favor the spread of *salmonellae* among reared animals, the use of untreated sludge to fertilize agricultural land and rendering of animal offal into frequently contaminated feed proteins (D'Aoust, 1997; 2001; Molbak *et al.*, 2006).

The European Union (EU) has set for the United Kingdom (UK) an annual target of 10% reduction in the prevalence of *S. Enteritidis* and *S. Typhimurium* in commercial egg laying holdings. To provide a baseline estimate for this target, data from commercial egg laying flocks were analyzed using Bayesian methods (Arnold *et al.,* 2010). The results indicated the prevalence of infected holdings for all *Salmonella* serovars was 18% (95% credibility interval 12-25%). For *S. Enteritidis* and *S. Typhimurium* only the prevalence was 14% (95% credibility interval 10-20%). These estimates were higher than previous analyses (11.9%), because the uncertainty caused by testing only one flock per holding and test sensitivity were taken into account. This analysis also supported the need for increased monitoring to provide future robust estimates. Such increased monitoring has been introduced under the 2008 UK National Control Plan for *Salmonella*.

Table 4. Prevalence of *Salmonella* in egg products in different country

Country (Reported Year)	Investigated Year	Sample	No. of Samples	% Positive	Serotype information (% of isolates)	Reference
Canada (1998)	Not Specified	Pooled egg products	21	0.00%		(Shaw *et al.*,1998)
Canada (1998)	Not Specified	Egg powders and liquid eggs	200	0.5%	*Salmonella* spp. (100%)	(Blais *et al.*, 1998)
(Japan (2006)	2003	Liquid whole eggs	14	0.00%	*S. Enteritidis* (100%)	(Kudo *et al.*, 2006)
		Liquid egg yolks	14	7.14%	*S. Enteritidis* (3.8%)	
Japan (2007)	Not Specified	Liquid eggs	627	0.96%	*S. Enteritidis* (100%) %	(Lapuz *et al.*, 2007)
Japan (2009)	1992 - 2002	Commercial liquid eggs	1327	8.1%	*S. Enteritidis* (50%) Wide variety of other serotypes	(Hara-Kudo and Takatori, 2009)
UK (2010)	Not Specified	Pooled raw shell egg mix	7641	0.13%	*S. Enteritidis*	(Gormley *et al.*, 2010a)
Pakistan	2009-2010	Eggshell	120	40%	*S. enteritidis* (83.33%)	(Akhtar *et al*. 2010)
		Egg interiors	120	8.33%	*S. typhimurium* (14.58%)	

2.4.1. Habitat of *Salmonella*

The primary habitat of *Salmonella* is the intestinal tract of farm animals, humans, birds, reptiles, and occasionally insects. Although they have also been found in spleen, liver, bile, mesenteric and portal lymph nodes, diaphragm, and pillar as well. As intestinal forms, the organisms are excreted in feces from which they may be transmitted by insects and other living creatures to a large number of places. Moreover, they may also be found in polluted water (Jay, 2000; Vieira-Pinto *et al.*, 2005).

Salmonella can survive for 9 months or more in the environment in sites such as moist soil, water, fecal particles and animal feeds, especially in blood-and-bone and fish meals (D'Aoust, 2001; Molbak *et al.*, 2006). The ubiquity of *salmonellae* in the natural environment, coupled with the intensive husbandry practices used in the meat, fish and shellfish industries and the recycling of offal and inedible raw materials into animal feeds, has favored the continued prominence of this human bacterial pathogen in the global food chain (D'Aoust, 1997).

European Union scientific committee concluded that the food categories that possibly pose the greatest hazard to public health include: raw meat and some meat products intended to be eaten raw, raw or undercooked poultry meat products, eggs and products containing raw eggs and unpasteurized milk and some milk products. Sprouted seeds, and unpasteurized fruit juices are also of concern (D'Aoust, 1997; Jay, 2000; Chiu *et al.,* 2004; Forshell and Wierup, 2006). Contamination of milk usually occurs after the milk leaves the cow, even though the organism can be excreted into the milk during the acute phase of the disease and occasionally by carrier animals (Radostits *et al.,* 1994). Moreover, *salmonellae* have been found in commercially prepared and packaged foods. Among the contaminated foods were cake mixes, cookie dough, cornbread mixes, coconut meal, salad dressing, mayonnaise, milk and other foods (Jay, 2000; D'Aoust, 2001; Molbak *et al.,* 2006).

2.4.2. Host Range of *Salmonella*

Salmonella have a wide variety of domestic and wild animal hosts. All members of the genus are considered to be potentially pathogenic, although serotypes may differ widely in their host range and the pathogenic syndromes that they produce. From the epidemiological point of view, *salmonellae* can be classified in to three main groups. The first group (human-adapted) comprises *S. Typhi, S. Paratyphoid A* and *S. Paratyphoid C,* which infect humans only and are spread directly or indirectly from person to person. The second group includes serotypes that are host-adapted for particular species of vertebrates. Included are *S. Gallinarum* (poultry), *S. Dublin* (cattle), *S. Abortus-equi* (horses), *S. Abortus-ovis* (sheep) and *S. Choleraesuis* (swine). Some of these are also pathogenic for human (especially *S. Dublin* and *S. Choleraesuis*). The third group (nonhost-adapted) contains the majority of the other *Salmonella* serotypes with no particular host preference that infect both humans and other animals (WHO, 1988; Jay, 2000; Forshell and Wierup, 2006).

2.4.3. Source of Infection and Transmission

Salmonellae are mainly transmitted by the fecal-oral route. They are carried asymptomatically in the intestines or gall bladder of many animals, and are continuously or intermittently shed in the faces (Radostits *et al.,* 1994; OIE, 2005; Forshell and Wierup, 2006). They can also be carried latently in the mesenteric lymph nodes or tonsils; these bacteria are not shed, but can

become reactivated after stress or immunosuppression. Fomites and mechanical vectors (insects) can spread *Salmonella* (OIE, 2005). Vertical transmission occurs in birds, with contamination of the vitelline membrane, albumen and possibly the yolk of eggs. *Salmonella* spp. can also be transmitted *in utero* in mammals (OIE, 2005).

Animals can become infected from contaminated feed (including pastures), drinking water or close contact with an infected animal (including humans). Birds and rodents can spread *Salmonella* to livestock. Carnivores are also infected through meat, eggs and other animal products that are not thoroughly cooked. Cats sometimes acquire *S. Typhimurium* after feeding on infected birds or spending time near bird feeders (OIE, 2005; Forshell and Wierup, 2006).

The trade of live animals within and between countries spreads *Salmonella*. Moreover, trade in contaminated animal feed products has also significantly contributed to the spread of *Salmonella* and several large outbreaks in humans have been traced back to contaminated animal feed. *Salmonella* is additionally spread between countries by humans as a result of food borne infections acquired abroad. The overall importance of these routes of transmission may reflect the prevalence of *Salmonella* contamination of food (including food of animal origin) in a particular country (Forshell and Wierup, 2006).

2.4.4. Pathogenesis

Salmonella produces different virulence factors that play an important role in its pathogenicity. These involve (1) the potential to invade the cell (2) a perfect lipopolysaccharide coat (3) to replicate intracellularly and (4) feasibly the secretion of toxins (Bhat *et al*., 2022). After entering the small bowel, *salmonella* must traverse the intestinal mucus layer before encountering and adhering to cells of the intestinal epithelium. *Salmonella* express several fimbriae that contribute to their ability to adhere to intestinal epithelial cells (Fabrega and Vila, 2013). Once across the intestinal epithelium, *salmonella* encounter another obstacle of innate immunity, the sub mucosal macrophage. *Salmonella* serotypes that cause systemic infection enter macrophages, again apparently by induced macropinocytosis, and subsequently activate virulence mechanisms that allow evasion of the microbiocidal functions of the phagocyte, permitting survival and replication in the intracellular environment.

Migration of infected phagocytes to other organs of the reticuloendothelial system probably facilitates dissemination of bacteria in the host (Ohi and Miller, 2001; Walpole *et al.,* 2021). The invasive strains that produce septicemia are able to escape destruction by the host and to multiply within the macrophages of the liver and spleen as well as intravascularly. The invasive abilities of some strains of *S. Typhimurium* are increased by the presence of genes carried on a plasmid. O-repeat units of the lipopolysaccharide prevent destruction within the blood stream. It is thought that they may mask determinants on the bacterial cell surface that would normally bind complement and activate it by means of the alternate pathway. This would reduce chances of chemotaxis, opsonization and phagocytosis. As a result there will be multiplication of the organisms in the body that subsequently leads to a severe endotoxaemia. Furthermore, these invasive *salmonella* secrete siderophores, which remove iron from iron-binding proteins of the host (Radostits *et al.,* 2007).

2.4.5. Carrier States

Because s*almonellae* are facultatively intracellular organisms that survive in the phagosome of macrophages, they can evade the bactericidal effects of antibody and complement. Thus, persistence of infection in animals and in the environment is an important epidemiological feature of salmonellosis. When an animal is infected with *S. Dublin* it may become a clinical case or an active carrier, passing organisms constantly or intermittently in the feces. It may also become a latent carrier with infection persisting in lymph nodes or tonsils but no *salmonellae* in the feces, or even a passive carrier, which is constantly picking up infection from pasture, but is not invaded so that when is removed from the environment the infection disappears (Radostits *et al.,* 2007; Wang *et al.,* 2020).

The importance of the latent carriers is that they become active carriers or even clinical cases under stressful conditions. Although all infected adults become carriers it is rarely for any length of time, and calves rarely become carriers. In sheep and cattle the carrier state may persist for as long as 10 weeks and in horses up to 4 months (Radostitis *et al.,* 2007). The intermittent fecal shedding that may follow the acute phase of human salmonellosis may be of short duration (convalescent carrier) or may persist for one or more years (chronic carrier) if the condition is not effectively treated. Carrier states are of concern to the food manufacturing and food service industries because of the perceived risk of cross contamination of foods by

infected food handlers and potentiating of food borne disease outbreaks. Although temporary exclusion of food handlers with non-typhoid diarrheal illness from work in sensitive areas is common practice, there is lack of unanimity on the need to exclude asymptomatic workers from their food-related duties and need to subject them to follow-up stool testing (Gunn *et al*., 2001).

2.5. Clinical Signs

The disease manifestations depend on the virulence of different *Salmonella* serovars; the number of *Salmonella* ingested and host immunity. Many *Salmonella* infections are opportunistic infection in compromised hosts. The majority of *Salmonella* infections in a herd over time are subclinical; the clinical infections are only the tip of the iceberg, even during outbreaks of clinical disease (Peek *et al*, 2018). A sudden change in herd resistance caused by concurrent disease, for example, fascioliasis, bovine virus diarrhea virus, nutritional stress, or severe weather can result in clinical disease (Wray and Davies, 2003).

Salmonellosis is manifested in animals in three major forms: enteritis, septicemia, and abortion. Fever, in appetence, and depression are commonly observed in acutely ill animals. Enteritis caused by *Salmonella* results in the passage of foul-smelling, watery feces, which may contain fibrin, mucus, and sometimes blood. When the enteric disease is severe, death may result from dehydration, electrolyte loss, and acid-base imbalance. Septicemic form often leads to abortion. *S. Dublin* has been associated with outbreaks of abortion in cattle, and several other serovars adapted to animal hosts have a particular association with abortion. The signs and lesions are not pathognomonic and in many cases, especially in poultry and pigs, *Salmonella* infections may be in apparent (Holschbach and Peek, 2018).

2.6. Diagnosis

Salmonella can be isolated either from tissues collected aseptically at necropsy or from faeces, rectal swabs, environmental samples, food products and feedstuffs. When infection of the reproductive organs, abortion or conceptus occurs, it is necessary to culture fetal stomach contents, placenta and vaginal swabs and, in the case of poultry, embryonated eggs. Individual samples for bacteriological tests should be collected as aseptically as possible by following the respective standards. Moreover, precautions should be taken to avoid cross contamination

of samples during transit and at the laboratory. Packages should also be kept cool and accompanied by adequate information (OIE, 2005).

Conventional cultural methods for the detection of food borne *Salmonella* spp. generally consist of five distinct and successive steps: pre-enrichment in nonselective media, selective enrichment in broth media, plating out on differential agar, biochemical screening and serological confirmation (Guyassa and Dima, 2016).

2.6.1. Pre-enrichment in Nonselective Liquid Medium

The number of *Salmonella* in faeces from asymptomatic animals, environmental samples, animal feed and food is usually low, and are often accompanied by considerably larger numbers of other *Enterobacteriaceae* or other families. Therefore, it is necessary to use pre-enrichment media to assist the isolation. Furthermore, pre-enrichment is necessary to permit the detection of sub lethally injured *Salmonella* (ISO 6579, 2002; 2017).

Pre-enrichment in a nonselective broth/liquid medium for 18-24h at 34-38^{o}C allows the small numbers of *salmonellae*, which may otherwise be killed by the toxic effect of enrichment media, to multiply, or it may help to resuscitate *salmonellae* that have been sub lethally damaged, e.g. by freezing, heating, exposure to biocides or desiccation. Pre-enrichment may not be the best method for isolating less vigorous *Salmonella* strains, such as the host-adapted strains, from faces because of overgrowth by competing organisms during nonselective pre-enrichment (D'Aoust, 2001; Elmerhebi, 2018). The use of short (6-8h) pre-enrichment for greater method brevity is not recommended because it fails to provide *salmonellae* with sufficient time to adapt to its new environment, repair cellular damage and actively grow to high numbers (D'Aoust, 2001).

2.6.2. Enrichment in Selective Liquid/broth Media

Enrichment media are liquid or semi-solid agar media that contain additives that selectively permit *salmonellae* to grow while inhibiting the growth of other bacteria (Sampson, 2021). Some, however, are also relatively toxic to certain serotypes of *Salmonella*, e.g. selenite inhibits *S. Cholerasuis*, and brilliant green is toxic to many strains of *S. Dublin*. Elevated temperatures have also been used to increase the selectivity of enrichment medium, and a

temperature of 43°C is used in some laboratories, although this may be inhibitory with some media, e.g. Tetrathionate and Rappaport-Vassiliadis at 43°C inhibit temperature-sensitive strains, especially *S. Dublin* and 41.5°C is now recommended for incubation of Rappaport-Vassiliadis broth. Selective motility enrichment may also be used to increase the sensitivity of *Salmonella* isolation and semi-solid enrichment media, e.g. modified semi-solid Rappaport-Vassiliadis or Diagnostic Semi-Solid *Salmonella* medium, may provide greater sensitivity. The formulation of the medium, temperature and duration of incubation, and the volume of the samples used to inoculate the medium, may all serve to improve the isolation rate, and these variables should always be taken into account. Examples of selective enrichment medium are sodium Tetrathionate, as in Muller-Kauffman broth, selenite cysteine (SC), Tetrathionate brilliant green (TBG) broth and Rappaport-Vassiliadis (RV) broths, or semi-solid Rappaport-Vassiliadis medium. Additions such as Ferrioxamine E may be added to selective media to enhance isolation of *Salmonella* from iron or nutrient-limited samples such as eggs, water or soil (D'Aoust, 2001; ISO 6579, 2002; 2017).

The concurrent use of two enrichment media where one medium is incubated at 41-43°C and the other at a more permissive temperature (35-37°C) maximizes detection of the target microorganism. These conditions accommodate fastidious *Salmonella* spp. that grow poorly or are inhibited at elevated temperatures and whose growth is hampered in highly selective enrichment media such as TBG and RV. Numerous studies on the reliability of enrichment conditions support the following decreasing order of effectiveness: TBG43 ≥ RV43 > TBG35 > SC35 (D'Aoust, 2000; 2001).

2.6.3. Plating Out and Identification

Enrichment cultures are plated onto selective agar media for the presumptive identification of *Salmonella* colonies on the basis of discriminating biochemical reactions. Standard plating out media include brilliant green agar (BGA), Brilliant green sulfa (BGS), Bismuth sulfite agar (BSA), Xylose Lysine Deoxycholate (XLD) and Hektoen enteric (Hek) agars that report on acid production from lactose and/or sucrose utilization through determinant color changes in the media. *Salmonella* spp. that typically produce hydrogen sulfide appear as charcoal black colonies with or without a black halo that produce a metallic sheen under reflected light.

The Rambach and SM-ID plating media produce discriminating color reaction in the presence of isolates that are galactosidase positive and that produce acid from propylene glycol (Rambach) and glucoronate (SM-ID). Plating media generally yield suspect colonies within 18-24hr incubation at 35-37°C, except BSA, which may require 48 h incubation for the development of presumptive *Salmonella* colonies. Comparative studies support the following ranking in decreasing order of effectiveness: BSA > BGS > BGA ≥ Rambach = SM-ID > XLD >Hek (D'Aoust, 2000; 2001; ISO 6579, 2002; 2017).

2.6.4. Confirmation

For confirmation, at least four-five presumptive (typical or suspect) *Salmonella* colonies were selected from every selective plating media. If the suspected colonies on each plate are fewer than five, all the colonies were selected. The selected colonies were streaked onto the surface of pre-dried nutrient agar plates, in a manner that was allowed well-isolated colonies to develop. Then the inoculated plates were incubated at 37ºC ± 1ºC for 24hr ± 3hr. The pure cultures on nutrient agar were used for biochemical and serological confirmation (ISO 6579, 2002; 2017).

2.6.4.1 . Biochemical confirmation

The use of lactose and/or sucrose, the production of H_2S and presence of lysine decarboxylase and urease are key determinants in the biochemical screening of presumptive *Salmonella* isolates (Soria and Bueno, 2016; ISO 6579, 2002; 2017). *Salmonellae* are facultatively anaerobic and, with few exceptions, all produce gas from fermentable sugars. In culture, nitrates are reduced to nitrites, and hydrogen sulfide is produced. Glucose and maltose are fermented, dulcitol and inositol are variably used, and sucrose is not fermented (Velge *et al.*, 2005)

2.6.4.2 . Serological confirmation

The three kinds of surface antigens, O antigens, H antigens, and Vi antigens, determine the reactions of the organisms to specific antisera. The detection of the presence of *Salmonella* O, Vi and H-antigens are done by the slide agglutination with the appropriate sera, from pure colon after auto-agglutinable stains have been eliminated. This method relies on the

antibody/antigen reaction between a test culture and commercially prepared antiserum (Popoff and Le Minor, 2001; ISO 6579, 2002; 2017).

2.7. Salmonellosis in Man

Salmonellosis become more prevalent in different countries as was the case with Europe. Despite the fact that the genus *Salmonella* contains over 2600 serovars, only 5–8 serovars cause the majority of human Salmonellosis cases in the United States. As per CDC, *Salmonella enteric* ser *Enteritidis* (24.7%), *S.* ser *Typhimurium* (23.5%), *S.* ser *Newport* (6.2%), and *S.* ser. *Heidelberg* were responsible for approximately 60% of human cases. That year, 04 serotypes accounted for 46.4% of non-human isolates. Main reason for infection in humans and other mammals is *S. enteric* which is responsible for 99% of overall infection (Mascellino, 2018). With respect to human disease, *Salmonella* serotypes can be divided into three groups that cause distinctive clinical syndromes, typhoid fever, bacteremia and enteritis (Santos *et al.,* 2001).

The non-typhoid *Salmonella* serotypes can cause protean manifestations in humans, including acute gastroenteritis, bacteremia and extra intestinal localized infections involving many organs (Chiu *et al.,* 2004). Within *Salmonella enteric* subspecies I (*Salmonella enteric* sub species *enteric*), the most common O-antigen sero groups are A, B, C1, C2, D and E. Strains within these sero groups cause approximately 99 % of *Salmonella* infections in humans and warm-blooded animals. Serotypes in other subspecies are usually isolated from cold blooded animals and the environment but rarely from humans (Velge *et al.,* 2005). Following ingestion of contaminated food or water, the pathogenesis of both typhoid and *Salmonella* enteritis begins with the intestinal phase, while only typhoid progresses to a systemic phase (Brown *et al.,* 2005).

Transmission of this disease within the human population is generally a result of poor sanitation of water and food supplies in developing nations. The broad host-range *Salmonella* serovars are prevalent within warm-blooded animal populations that make up the human food supply and bacterial transmission generally results from consumption of raw or under cooked food products (Jones, 2005).

2.8. Salmonellosis in Animals

Salmonella serotypes have a broad host range and prevalent in the warm blooded animal population including rodents (Ferrari *et al.,* 2019). Reptiles kept as pets, such as turtles, guanas, other lizards and snakes, are often identified as non-food sources of infection (Maslanka *et al.,* 2023). Some serotypes are highly adapted to animal hosts, such as *S. Gallinarum* in poultry and *S. Abortus-ovis* in sheep. Many non-typhoidal *Salmonella* strains such as *S. Typhimurium* and *S. Enteritis's* infect a wide range of animal host including poultry, cattle and pigs (Ohl and Miller, 2001). These serotypes generally cause self-limiting gastrointestinal infections usually less severe than enteric fever in humans. However, they also have the capacity to produce typhoid-like infections in mice and in humans or asymptomatic intestinal colonization in chickens (Velge *et al.,* 2005).

2.8.1. *Salmonella* in Food Animals in Ethiopia

Foods of animal origin, especially poultry and poultry products, including eggs, have been consistently implicated in sporadic cases and outbreaks of human salmonellosis, and chicken products are widely acknowledged to be a significant reservoir for *Salmonella* (Panisello *et al.,* 2000).

In Ethiopia, there is no more *Salmonella* serotype and antimicrobial resistance surveillance and monitoring system, therefore the available information are fragmented and made available through individual publications. One study, conducted on chicken and different chicken products in Ethiopia indicated the presence of different serotypes of *Salmonella* (Molla and Mesfin, 2003). In that study out of the total 80 *Salmonella* isolates, 8 different serotypes were identified of which *S. Braenderup* was the most frequent followed by *S. Typhimurium var. Copenhagen*, *S. Anatum*, *S. Kottbus* and *S. Typhimurium*. Other serotypes isolated include *S. Bovismorbificans*, *S. Hadar* and *S. Infantis*, *S. Braenderup, S. Anatum* and *S. Newport* appear to be the major *Salmonella* serotypes associated with chicken meat and chicken meat products around Addis Ababa (Tibaijuka *et al.,* 2002; Zewdu, 2008).

2.8.2. *Salmonella* in Poultry

The industrialization and globalization of poultry and egg production have been linked to the introduction of *Salmonella* in poultry and subsequently in egg production (Antunes, *et al.*, 2016). *Salmonella* can infect poultry from a variety of sources, including as birds, feed, rats, and other vehicles. With the exception of infections brought on by the host-adapted serovar Pullorum/ Gallinarum, which are rare in terms of clinical disease, the majority of infections are significant as possible causes of food poisoning in humans. During the slaughter process, poultry products can get contaminated at various stages. This can occur from contact with infected products or from excrement during the evisceration process (Hans and Dean, 2006).

2.8.3. *Salmonella* in Eggs

Salmonella is a leading cause of food-borne illness in many countries with eggs and poultry being important vehicles of transmission. During the past two decades *S. Enteritis's* has become a leading serotype causing human infections, with hen eggs being a principal source of the pathogen (Shaji *et al.*, 2023).

Bacterial infections of shell eggs and its content such as *Salmonella* spp., especially *S. Enteritis's* can occur in two different ways: either vertically or horizontally (Gast *et al.*, 2024). In vertical transmission, *Salmonella* are introduced from infected reproductive tissues to eggs prior to shell formation. Horizontal transmission is usually derived from fecal contamination on the egg shell. It also includes contamination through environmental vectors, such as farmers, pets and rodents. They may be able to contaminate egg contents by migration through the eggshell and membranes. Such a route is facilitated by moist eggshells, storage at ambient temperature and shell damage by *Salmonella* (Yan, 2021).

Table 5. Prevalence of *Salmonella* in egg products in Ethiopia

Area	Investigated Year	Sample type	No. of Samples	No. of Positive	% Positive	Reference
Kombolcha	2009-2010	Shell	400	25	6.3	Minte *et al.*, 2011
		Content	400	27	6.8	
Addis Ababa	Not Specified	Whole egg	384	20	5.21	Bayu *et al.*, 2013
Mekele	Not Specified	Shell	156	24	15.38	Dawit *et al.*, 2016
		Contents	156	13	8.33	
Haramaya area	2012-2013	Shell	300	16	5.3	Jelalu *et al.*, 2016
		Content	300	0	0.00	
Alage	2013-2014	Shell	196	15	7.7	Tsegaye *et al.*, 2016
		Content	196	11	5.6	
Haramaya Univ.	Not Specified	Shell	384	9	2.4	Tessema *et al.*, 2017
		Content	384	2	0.5	
Mizan Teferi	2017-2018	Shell	366	23	6.3	Hailu *et al.*, 2021
		Content	366	4	1.1	

2.9. Health and Economic Impact of Salmonellosis

Salmonella is one of the eight microorganisms in the European Union Zoonosis Monitoring Directive, which shows a disease considered to have a high impact on human health in the Union (Jong and Kedah, 2006). Poultry products have always topped the incidence of salmonellosis in many developing countries including India, Egypt, Brazil and Zimbabwe (Henson, 2003) and is the most seriously perceived food risks in chicken meat, even in the developed countries (Young, 2001). *S. Enteritis's* is transmitted to the human food supply through eggs of hens that appear healthy (Porwollik *et al.*, 2005). Contamination with *Salmonella* in poultry products can occur at multiple steps along the food chain, which includes production, processing, distribution, retail marketing, handling and preparation (Cui *et al.*, 2005).

The estimated annual costs in 1998 and 2003 of medical care and lost productivity due to food borne *Salmonella* infections in the United States were 2.3 and 2.9 billion dollars (ERSUSDA, 2003). In the year 2000, 16,983 laboratories confirmed cases of salmonellosis were reported in the United Kingdom, most commonly associated with the consumption of chicken and undercooked egg dishes (UK zoonosis report, 2000). In the year 2001, China recorded that

17.9 % of the total food poisoning was caused by *Salmonella* spp. (FAO/WHO, 2004). Apart from the impact on the health of the individuals, economic losses on international and national trade due to *Salmonella* in the poultry products and product recalls have direct economic and public perception effects on the processing industries (Kramer *et al.,* 2005).

In Ethiopia as in other developing countries, it is difficult to evaluate the burden of salmonellosis because of the limited scope of studies and lack of coordinated epidemiological surveillance systems. In addition, under-reporting of cases and presence of other diseases considered to be of high priority may have overshadowed the problem of salmonellosis (Kebede *et al*., 2016). Until 1981, comprehensive study on invasive *Salmonella* was not conducted in Ethiopia (Gedebou and Tassew, 1981).

2.10. Antimicrobial Resistance o*f Salmonella*

Salmonella display high natural susceptibility levels to the most commonly used antibacterial agents (Cabrera *et al.,* 2004). Conventional antimicrobial agents, such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole, had been the drug of choices in the treatment of salmonellosis before the 1980s (Chiu *et al.,* 2004). However, the occurrences of isolated *Salmonella* strains showing resistance to one or more antibacterial agent have steadily increased worldwide, probably due to continuous antibiotic pressure (Cabrera *et al.,* 2004; Chiu *et al.,* 2004; Schroeter *et al.,* 2004; Agustina *et al.,* 2005). This is an important public health problem that may be related to therapeutic failure.

Moreover, the problem is especially relevant in developing areas, where the lack of economic resources does not allow a wide antibacterial armamentarium (Cabrera *et al.,* 2004). Extended-spectrum cephalosporin's and fluoroquinolones have been suggested as appropriate alternative agents in the treatment of infections caused by such multidrug-resistant *Salmonella* serotypes; however, since 1991, outbreaks or cases of infections caused by *Salmonella* serotypes resistant to extended-spectrum cephalosporin's or fluoroquinolones have been increasingly reported (Chiu *et al.,* 2004).

The emergence of antimicrobial resistance in *Salmonella* is complicated because the use of antibiotics for therapeutic purposes in veterinary medicine and as growth promoters in animal

feed, thus presenting a potential risk to public health from zoonotic infections (Schroeter *et al.*, 2004; Agustina *et al.*, 2005). In addition, pet animals such as frogs and turtles and their water environment were shown to carry multidrug-resistant *Salmonella* strains, which could subsequently cause infections in humans. Therefore, to curb the resistance problem in *Salmonella*, it has been suggested that inappropriate use of antimicrobial agents in food animals should be prohibited (Chiu *et al.*, 2004).

2.11. Drug Susceptibility Patterns of *Salmonella* in Ethiopia

Studies conducted in Ethiopia on Salmonellosis indicts increase in the antimicrobial resistance of *Salmonella* to commonly used antimicrobials in both public health and veterinary sectors (Abebe *et al*., 2014; Addis *et al*., 2011; Liyuwork *et al*., 2013) in Debrezeit determined from health slaughtered cattle indicated that five different serovars isolation were *S. mishmarhaemek* (48%), *S. Typhimurium* (20%), *S. Enteritidis* (12%), *S. Guildford* (12%), *S. Dublin* (48%). Both strains *S. mishmarhaemek* and *S. Typhimurium* showed multiple resistances to ampicillin, sulfamethoxazole and tetracycline. (Endrias *et al*., 2009), *Salmonella* isolates belonging to *S. Newport, S. Typhimurium, S. Infantis, S. Bovismorbificans, S. Anatum, S. Zanzibar, S. Kottbus, S. Saintpaul* were found to be susceptible to all antimicrobials tested. However, *Salmonella* isolates (32.7%) belonging to *S. Braenderup* 83.3% Ampicillin, spectinomycin, Streptomycin, *S. Hadar* 37.5% Streptomycin, Tetracycline, *S. Dublin, S. Haifa* 100% Ampicillin, Streptomycin, Tetracycline, and *S. Kentucky* 100% Ampicillin, Ciprofloxacin, Gentamicin, Nalidixic acid, Streptomycin.

2.12. Zoonotic Implication

National and global epidemiological registries continue to highlight the importance of *Salmonella* spp. as one of the leading cause of food-borne bacterial zoonotic disease in humans (D'Aoust, 2000; Ferrari *et al*., 2019). It is noteworthy that the problem of human salmonellosis from the consumption of contaminated foods generally remains on the increase worldwide (D'Aoust, 2000). The cumulative losses of salmonellosis are due to productivity losses and absenteeism, pain and suffering, lost leisure time and medical costs.

Various clinical forms of *Salmonella* such as gastroenteritis, bacteraemia and other systemic abnormalities can occur in veterinarians working with *Salmonella*-infected animals (Radostits *et al.,* 1994). Moreover, cutaneous salmonellosis has been reported in veterinarians attending to infected cattle at the time of parturition. The disease was characterized by pustular dermatitis from which *S. Virchow* and *S. Dublin* were isolated. Similarly, pustular dermatitis caused by *S. Stanley* developed on the arm of a veterinary surgeon after the delivery of a dead bovine calf. Unlike the previous reports, the veterinarian did not develop any systemic symptoms and made a full recovery (Lazarus *et al.,* 2007).

2.13. Treatment

Treatment of non-typhoidal *Salmonella* infection is different from typhoidal infection. In treatment of non- typhoidal *Salmonella* infection antibiotics should not be used routinely, as used in typhoid. Antibiotic should be only used if required as most infection with non-typhoidal *Salmonella* is self-limiting type and duration of diarrhea and fever are not much affected by use of antibiotics. Additionally antibiotic therapy can increase relapse of infection and also prolong the duration of gastrointestinal carrier states. The main treatment should be aimed at correcting dehydration that may arise due to prolonged diarrhea by fluid and electrolyte replacement (www.webcan.gogleuserconten.com).

Antibiotics are used for the prevention, treatment and control of infectious diseases as drugs for people and animals. Excessive use of antibiotics can, however, cause antibiotic resistance. The role of cattle in emerging and propagating medicinally resistant antimicrobial bacteria and the proliferation of antimicrobial resistance are poorly understood among humans (Fall-Niang *et al*., 2019). The development of antibiotic-resistant *Salmonella* primarily results from the use of antibiotics for feed-based animals to facilitate growth and antibiotics in veterinary medicine for the treatment of bacterial infections (Hyeon *et al*., 2011).

2.14. Prevention and Control of Salmonellosis

Prevention of salmonellosis by the implementation of hygiene measures is difficult and use of antibiotics may give rise to the emergence of resistance problems (Mastroeni and Menager, 2003). Reducing *Salmonella* prevalence requires a multi-hurdle approach at all stages of breeding, hatching, grow-out, transportation and processing. Attenuated DNA recombinant

live *Salmonella* vaccines (Kang *et al.,* 2002), combined with comprehensive control strategy in animals, feed and animal food stuffs will help to reduce salmonellosis. Additional measures to control secondary contamination could be prevention of contamination by cleaning and disinfection, hygiene of personnel and proper processing (Sinell, 1995). Growth of microorganisms in meat and poultry products can be controlled by maintaining a cold chain at 10°C, especially for *Salmonella* during transport and storage (NidaUllah *et al.,* 2016). Vaccination does not offer complete protection of the birds against infection. An effective way to control *Salmonella* in eggs is by preventing the vertical spread of *S. Enteritis's* between different generations of birds. The principle is a top down approach, eliminating *Salmonella* from the top of the production pyramid and downwards by test and slaughter (Hans and Dean, 2006).

The post-harvest control level of *Salmonella* in infected/contaminated eggshells can be reduced by decontamination of the surface of the shell by heating, irradiation or other means. Whole eggs should be pasteurized by the process of heating to 60 °C (140 °F) for 3.5 minutes. Liquid egg white is pasteurized at 56.7 °C (134 °F) for 3.5 minutes or 55.6 °C (132°F) for 6.2 minutes. Liquid egg yolk is pasteurized at 61.1 °C (142 °F) for 3.5 minutes or 60.0°C (140 °F) for 6.2 minutes. In recent years, pasteurized shell eggs have emerged. The process involves pasteurization of the shell egg in water baths for extended periods of time (hours) to achieve a temperature in the center of ~55 °C. Up to a 7-log reduction in *Salmonella* counts in the interior has been documented by this method. Special attention should be given to the preparation and handling of those foodstuffs that have caused a large proportion of outbreaks in recent years, namely mayonnaise, cold desserts and pre-cooked savories (Hans and Dean, 2006).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

Konta zone is one of the six zones in the SWEPRS. The study was conducted in Ameya town which is located in the Konta zone, located in between 6º, 46'-47',26'' North latitude and 36º, 32'-36',87'' East longitude at south western part of Ethiopia. Ameya the capital of Konta zone is found at a distance of 465 km away from the capital city of Ethiopia (Addis Ababa), 225 km from the regional town Bonga, and 372 km from Hawassa and 104 km away from Jimma town. Konta zone is bordered with Oromia Region Jimma zone in North, Dawuro zone in east, Kaffa zone in west, South Omo zone in the South and Gofa zone in the South east direction. Agro ecologically consists of 40%, lowland (790-1500m.a.s.l), 54% midland (1500-2300m.a.s.l) and 6% highland (>2300m.a.s.l). The mean annual temperature and rainfall varies from 15.1-36°Cand 1200-2290 (1745) mm respectively (KZADO, 2022).

According to Konta zone Finances and economic development office report 2022 the total Human population of the Konta zone is 220,280 and of which 108,649 are males and 111,631 are females. Among this rural inhabitants are 185,090 (84.02%) and of which males are 91,935 females are 93,155. Konta zone with an area of 238,163 square kilometer, the population density is 51.4 people per kilometer square and peoples are sparsely settled though high variation in between highland and low land (KZFEDO).

The district comprises the population of 398,666 poultry and poultry related diseases (KZADO, 2022). As a result, this study site is selected purposively because of first road accessibility and availability of farm and market second based on the severity of food borne disease problem in human and animals in the area (KZADO, 2022).

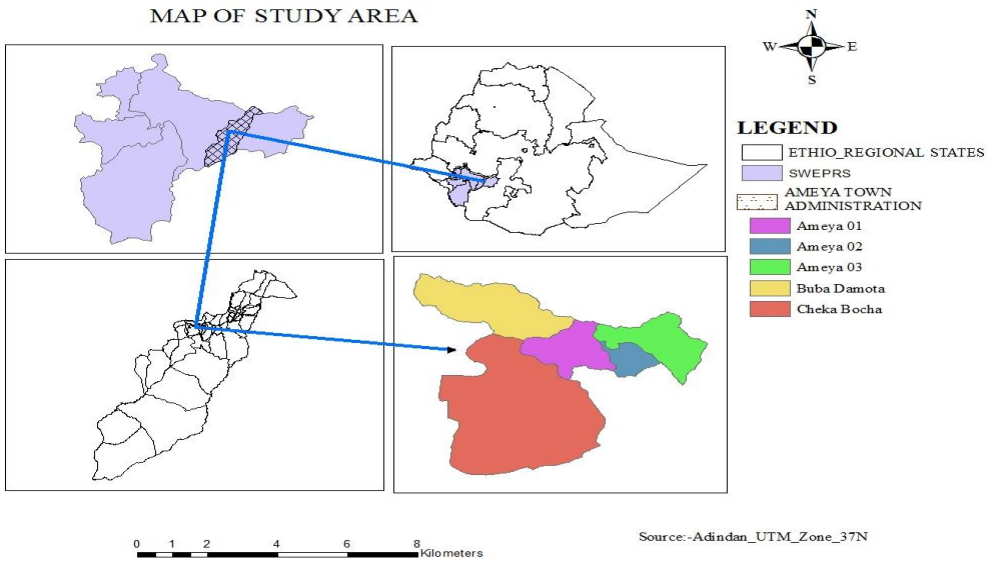

Figure 1. Geographic map of the study area

3.2. Study Design, period and Sampling

Cross-sectional study was conducted to determine the prevalence, antimicrobial susceptibility and its associated risk factors of *Salmonella* on raw chicken eggs from three breed groups (local, cross and exotic) of layer chickens from Ameya poultry farms and local markets (directly from the farmers in Konta-Ameya) from March, 2023 to April, 2024.

Sample size determination: The sample size required for this study was determined based on the expected prevalence of *Salmonella* and the desired absolute precision. There is no previous study on *Salmonella* in raw chicken eggs in the study area. Therefore, 50% expected prevalence with 95% desired confidence interval and 5% absolute precision was used and the formula stated by Thrusfield (2005) was used to determine sample size which was indicated below:

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

Where:
N = number of sample size.
P = expected prevalence (50%) = 0.5
d = marginal error between the samples and population (5%) = 0.05
Z_α = the standard normal deviation corresponding 95% of confidence level = 1.96

$$N = \frac{(1.96)^2(0.5)\ (1-0.5)}{(0.05)^2} = 384$$

According a total of 384 eggs was calculated; 192 eggs from poultry farms of egg storage and laying place and 192 eggs from Ameya open market directly from the farmer egg sellers (one egg from one egg seller) were collected. Forty-eight eggs from open market and forty-eight eggs from selected poultry farms were collected once per three weeks using a simple random sampling technique. Eggs were collected in sterile plastic bags and an aseptic procedure was strictly adopted during collection. Samples of collected eggs from the study area were individually placed into a sterile plastic container in an ice box and were transported to the Mizan Regional Veterinary Laboratory. Samples were processed within four hours upon arrival.

3.3. Sample Processing

The sterile plastic bags containing selected eggs were open with scissors and the samples processed immediately. Conventional cultural swab technique was used to sample the shell surface of the intact eggs. Sterile cotton swabs dipped into sterile buffered peptone water was used to swab the entire surface area of the egg shell and incubated in 37°C for 16-18 hrs.

The same eggs from which shell sample collected were used for interior (egg content mixture) sampling. The egg's surface was sterilized by 70% alcohol followed by flaming and then cracked with a sterile scalpel blade. The isolation was conducted utilizing the conventional methods for the detection of *Salmonella* following the standard guidelines from ISO 6579, 2002; 2017 (Figure 2).

3.4. Isolation, and Identification of *Salmonella*

3.4.1. Non-selective Pre-enrichment

The swabs were directly inoculated into 10 ml BPW in screw capped bottles and incubated at 34°C- 38°C for 16-18 hrs. Each 25 ml of the egg’s content was inoculated into 225 ml of BPW (1:9) and homogenized for two minutes with stomacher. After mixing thoroughly, the samples were incubated at 37°C for 16-18hrs (ISO 6579, 2002; 2017).

3.4.2. Selective Enrichment

Tetrathionate and Rappaport-Vassiliadis *Salmonella* enrichment broth were used for Selective Enrichment of all samples. From the pre-enrichment broth after incubation and thoroughly shaking, 0.1ml of the broth was transferred into a tube containing 10ml of Rappaport-Vassiliadis medium (RV broth). Another 1ml of the pre-enrichment broth was transferred into a tube containing 10ml of Muller Kaufman Tetrathionate novobiocin broth (MKTTn broth). The inoculated RV broth was incubated at 41.5 °C ± 1 °C for 24 ± 3 hours and the inoculated MKTTn broth at 37 °C ± 1 °C for 24 ± 3 hours (ISO 6579, 2002; 2017).

3.4.3. Plating Out and Identification

Xylose Lysine Deoxycholate (XLD) agar and brilliant green (BGA) agar plates were used for plating out and identification purpose. A loop-full of inoculums from RV and MKTTn broth cultures were transferred and streaked separately onto the surface of XLD agar and BGA plates. The plates were incubated at 37°C ± 1°C for 24 ± 3hours. After proper incubation, the plates were examined for the presence of typical and suspected *Salmonella* colonies, which on XLD agar were pink with a darker center and a lightly transparent zone of reddish color due to the color change of the indicator where as lactose positive *salmonellae* are yellow with or without blackening. Typical colonies of *salmonella* on Brilliant Green Agar (BGA) are pink, 1mm to 2mm in diameter and cause the color of medium to change to red. Four to five *Salmonella* presumptive colonies selected from the selective plating out media were transferred or streaked on to the surface of pre-dried nutrient agar plates and incubated at 37°C for 24 hrs for further confirmatory tests. Confirmation was done by using biochemical test according to ISO 6579, 2002; 2017 (Appendix 6).

3.4.4. Biochemical Confirmation Tests

The Triple sugar iron agar slant was prepared with a thick butt. A loop full culture of pure growth from nutrient agar plates stabbed into the butt and streaked on the slant and was incubated for 24 hours at 37°C. Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas production (bubbles) and formation of hydrogen sulfide (blackening of the agar) (Table 6).

Table 6. Observation of TSI agar for presence of *Salmonella*

Area of reaction	Result	Interpretation
Butt	Yellow	Glucose positive (glucose used)
	Red or unchanged	Glucose negative (glucose not used)
	Black	Formation of hydrogen sulfide
	Bubbles or cracks	Gas formation from glucose
Slant surface	Yellow	Lactose and/or sucrose positive (lactose and/or sucrose used)
	Red or unchanged	Lactose and sucrose negative (neither lactose nor sucrose used)

The hydrolysis of urea releases ammonia and production of ammonia increases the pH of the medium that change color of phenol red (pH indicator) to rose pink, and later to moderate red. The basal medium will be sterilized by autoclaving at 121°C for 15 minutes. When it has cooled to about 50°C, 100ml of a 20 percent solution of pure urea previously sterilized by filtration was added and poured into test tubes. The isolates were inoculated into the urea to determine urease production. The inoculated tubes were incubated at 37°C for up to 96 hours. The observation was made at an interval of 4, 24, 48 and 96 hours. Urease positive cultures changed the color of the indicator to red *(*Appendix 6).

Simmons's citrate agar was sterilized by autoclaving at 121°C for 15 minutes at 15 lb. pressure and cooled for slant formation. The strains were cultured on the prepared Simmons's citrate agar medium, incubated at 37°C for 48 hours and observation was recorded. Opacity and change in color of bromothymol from green to blue indicated a positive reaction.

The Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Types and proportion of fermentation products produced by anaerobic fermentation of glucose is one of the key taxonomic characteristics which help to differentiate various genera of enteric bacteria. Mixed acid fermentation is one of the two broad patterns, 2-3- butanediol fermentation being another. In mixed acid fermentation, three acids (acetic, lactic and succinic) are formed in significant amounts. The mixed acid pathway gives 4mol of acidic products (mainly lactic and acetic acid), 1 mol of neutral fermentation product (ethanol), 1 mol of CO_2, and 1 mol of H_2 per mol of glucose fermented these large amounts of acid results significant decrease in the PH of the medium below 4.4. This is

visualized by using pH indicator, methyl red (p-dimethylaminoaeobenzene-O-carboxylic acid), which is yellow above pH 5.1 and 40 red at pH 4.4. The pH at which methyl red detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media. Thus, to produce a color change, the test organism must produce large quantities of acid from carbohydrate substrate being used. Procedure for Methyl Red (MR) Test MR-VP broth is used for both MR test and VP test. Only the addition of reagent differs, and both tests are carried out consecutively (Appendix 6I).

Indole is a nitrogen-containing compound that can be formed from the degradation of the amino acid tryptophan by certain bacteria. Tryptone broth was used as a substrate because it contains much tryptophan. The indole reacts with aldehyde compound of Kovac's reagent and forms red colored compound that is more soluble in alcohol. For Indole test Tryptone broth was prepared and the ingredients was dissolved in distilled water, dispensed in test tubes and sterilized by autoclaving at 121°C for 15 minutes. The tubes of the medium was inoculated with test isolates using sterile platinum loop and incubated at 37°C aerobically for up to 96 hours. Finally, 0.5 ml of Kovac's reagent was added to each of the inoculated and un-inoculated controls. The tubes were shaken gently and the results were recorded. Positive results were indicated by the development of red color in the alcoholic layer of the reagent and no color in the control tube (ISO 6579, 2002; 2017).

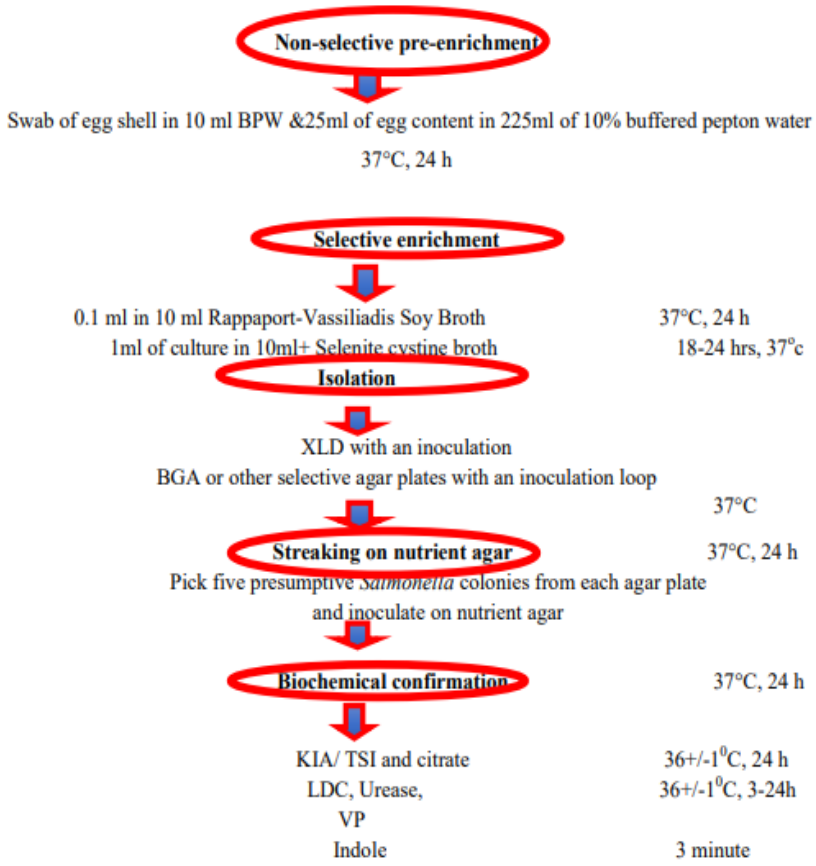

Figure 2. Procedure of isolation of *Salmonella* from raw chicken egg

Source: ISO 6579, 2002: WHO, 2003

3.5. Antimicrobial Susceptibility Testing

3.5.1. Disk Diffusion Testing

The antimicrobial susceptibility testing was done by using Kirby Bauer agar disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2018). The pure *Salmonella* isolates confirmed by the biochemical testing procedure as described in ISO 6579 was tested for antimicrobial susceptibility. The antibiotics to be used were selected among the currently available and commonly used chemotherapeutic agents for treatment of *Salmonella* infection in human and animals such as: Amoxicillin (AMX)20µg, Ampicillin (AML)10µg, Erythromycin (ERY)15µg, Gentamycin (GEN)10µg, Ceftriaxone (CRO)30µg,

Nalidixic acid (NA)30µg, Spectinomycin (SPC)100µg, Tetracycline (TET)30µg, Clindamycin (CLN)2µg, Kanamycin (KAN)30µg (OXOID) (Table 7).

3.5.1.1. Preparation of Mueller-Hinton agar

The Muller-Hinton agar was prepared as per the instructions provided by the manufacturer. After autoclaving the media at 121°C for 15 minutes, it was cooled to 50°C and approximately 30 ml to 50 ml was poured into the 15 x 150 mm Petri dishes. The depth of the agar in the Petrifies was maintained approximately at 4 mm. The freshly prepared plates were used on the same day (Appendix 6.).

3.5.1.2. Turbidity standards (McFarland) for inoculums preparation

A McFarland 0.5 turbidity standard was prepared as per the standard guidelines described by the Clinical and Laboratory Standards Institute. A volume of 0.5 ml of a 1.175% (wt./vole) barium chloride dehydrate ($BaCl_2.2H_2O$) solution was added to 99.5ml of 0.18 moll/L (1% vole/vole) sulfuric acid with constant stirring to maintain the suspension. The turbidity standard will be then aliquot into test tubes of 4 ml each, identical to those used to prepare the inoculums suspension. McFarland standards then were stored in the dark at room temperature (22°C to 25°C). Before each use, the standards were shaken well, mixing the fine white precipitate of barium sulphate in the tube.

3.5.1.3. Inoculums preparation

At least 3-5 well isolated colonies of the same morphological type were selected from the agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 4ml tryptic soya broth. The broth culture was either directly adjusted to the McFarland standards or by incubation at 35°C until it achieved or exceeded the turbidity of the 0.5 McFarland standards (usually 2-6 hours). The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to the point of the 0.5 McFarland standards (CLSI, 2018).

3.5.1.4. Inoculation of test plates

Optimally within 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the adjusted suspension. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level. This removed excess inoculums from the swab. The dried surface of a Muller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculums. As a final step the rim of the agar was swabbed. The procedure was done under laminar flow to avoid contamination. The lid was left ajar for 3-5 minutes but no more than 15 minutes, to allow for any access surface moisture to be absorbed before applying the drug impregnated disks (CLSI, 2018).

3.5.1.5. Application of disks to inoculated agar plates

The predetermined batteries of antimicrobial disks were dispensed onto the surface of the inoculated agar plate. Each disk was pressed down individually to ensure complete contact with the agar surface. The disk placed in the agar surface was not closer than 24mm from center to center. A total of six disks were placed on one 150mm plate. The plates were inverted and placed in an incubator set to 35°C within 15 minutes after the disks were applied (CLSI, 2018).

3.5.1.6. Reading plates and interpretation of results

After 16-20 hours of incubation, each plate was examined. The resulting zone of inhibition was uniformly circular with a confluent lawn of growth. The diameters of the zones of complete inhibition (judged by the unaided eye) were measured. Zones were measured to the nearest whole millimeter, using sliding calipers, which is held on the back of the inverted Petri plate. The petriplates were held a few inches above a black, non-reflecting background. Transmitted light from the colony counter is used to examine the zones for light growth wherever indicated, within apparent zones of inhibition. The zone margin was taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, was ignored. The sizes of zones of inhibition were interpreted by

referring to zone diameter interpretive standards from CLSI, 2018) and the isolates were reported as susceptible, intermediate or resistant to the agents that have been tested.

Table 7. Zone Diameter and Microbial inhibition concentration for *Enterobacteriaceae.*

No	Antimicrobial Agent	Disc Code	Potency	Resistant	Intermediate	Susceptible
1	Amoxicillin	AMX	20µg	≤13	14-17	≥18
2	Ampicillin	AML	10µg	≤13	14-16	≥17
3	Erythromycin	ERY	15µg	≤13	14-22	≥23
4	Tetracycline	TE	30µg	≤11	12-14	≥15
5	Kanamycin	K	30µg	≤13	14-17	≥18
6	Ceftriaxone	CRO	30µg	≤19	20-22	≥23
7	Spectinomycin	SPC	100µg	≤11	12-14	≥15
8	Nalidixic acid	NA	30µg	≤13	14-18	≥19
9	Clindamycin	CLN	2µg	≤14	15-20	≥21
10	Gentamicin	CN	10µg	≤12	13-14	≥15

Source: CLSI, (2018).

3.6. Questionnaire Survey

A questionnaire survey was pretested and administered to interviewee 40 egg sellers in the market and 35 farmers and consumers from different kebeles to assess potential factors favoring contamination of egg with *salmonella* species. Information about egg storage, transportation, and preparation and utilization patterns of chicken eggs was collected in the study area (Appendix 2). Five kebeles were included 7(seven) farmers and consumers were selected from each kebeles. A random sampling of households was done by using lists of households visited by health extension workers and Agricultural extension workers. From selected households who are responsible for food preparation and purchaser were interviewed using semi-structured questioners.

3.7. Data Management and Analysis

Data management was carried out using Microsoft Office Excel 2010 used for data entry, and these data were analyzed using SPSS software statistical package version 27.0 (IBM SPSS statistics) was used to generate descriptive statistics such as percentage and proportion was used to describe samples detected positive to *Salmonella* isolated from the total sample analyzed by sources of samples, sample type and samples from different breeds. It was generated using the procedure of frequency and expressed in percent. The Pearson's chi-square (X^2) test was used to determine the difference in levels between study groups and the association between the prevalence of *salmonella* and associated factors (sources, breeds and type of egg sample) or variation of prevalence. P-value of less than 0.05 was considered to determine statistically significant differences in all cases.

3.8. Ethical Consideration

The protocol for field studies, the collection of egg samples from open markets and poultry farms and materials were approved by the animal research ethics committee and the letter of clearance was obtained from Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) with ethical clearance Reference Number: A16/May/2024. The data was collected after verbally informed consent is made with all study participants. All the rights of privacy and confidentiality of participants were protected.

4. RESULTS

4.1. Prevalence of *Salmonella*

An overall prevalence of 8.85% *Salmonella* contaminated eggs were found both in egg contents and eggshells of 384 samples. From this, 6.77% and 2.08% were from egg shells and egg contents respectively. Moreover, the prevalence of *Salmonella* in the poultry farm was 6.25%, while it was 11.46% in the market (Table 8).

Table 8. Prevalence of *Salmonella* in eggs by source of samples

Source of sample	No. examined	Number of positives			Prevalence (95%CI)
		Eggshell	Egg content	Total	
Farm	192	11	1	12	6.25 (2.8-9.7)
Market	192	15	7	22	11.46 (7.0-16.0)
Total	384	26	8	34	8.85 (6.0-11.7)

The prevalence of *Salmonella* in eggshells from open markets was not significantly different ($p>0.05$) from that of *Salmonella* on eggshells from poultry farms. However, there was a significant difference ($p<0.05$) in prevalence between the contents of eggs purchased from the open market and egg contents obtained from the poultry farm (Table 9).

Table 9. The prevalence of *Salmonella* between farm and market (sample sources)

Sample type	Farm (n=192)	Market(n=192)	Total	X^2	p-value
Eggshell	11(5.73)	15(7.81)	26(6.77)	0.660	0.417
Egg content	1 (0.52)	7(3.65)	8(2.08)	4.596	0.032
Total	12(6.25)	22(11.46)	34(8.85)	3.22	0.072

The study's findings regarding the prevalence of *Salmonella* revealed that it was comparatively more common in egg shells (6.77%) than in egg contents (2.08%). As a result, according to Table 10, this difference was statistically significant ($p=0.002$).

Table 10. The prevalence of *Salmonella* in egg shell and egg contents

Sample type	No. of positive	Prevalence 95% CI	X^2	p-value
Egg shell (384)	26	6.77 (4.3-9.3)	9.971	0.002
Egg contents (384)	8	2.08 (0.7-3.5)		
Total	34	8.85 (6.0-11.7)		

A higher prevalence of *Salmonella* was recorded in samples from local breeds compared with crossbreeds and exotic breeds having a prevalence of 11.59%, 8.15% and 4.71% respectively. There was a statistically significant difference between eggshells of different breeds ($p<0.05$). However, there was no statistically significant difference between egg contents of different breeds. ($p>0.05$) (Table 11).

Table 11. Over all prevalence of *salmonella* based on samples in different breeds

Sample	Sample Breed				X^2	P-Value
type	Local(164)	Cross(135)	Exotic(85)	Total(384)		
Egg shell	16(9.76%)	9(6.67%)	1(1.18%)	26(6.77%)	6.532	0.038
Egg content	3(1.83%)	2(1.48%)	3(3.53%)	8(2.08%)	1.163	0.559
Total	19(11.59%)	11(8.15%)	4(4.71%)	34(8.85%)	3.11	0.182

The specific prevalence of *Salmonella* detected from the samples from different breed sources. Among the 19 local breed chicken egg samples positive for *Salmonella*, 16 (84.21%) were detected from Eggshells and only 3 (15.79%) were detected from Egg contents (Table 12).

Table 12. The prevalence of *Salmonella* in eggs by different breed sources

Breed	Samples examined	No. of positives			Prevalence (95%CI)
		Egg shell	Egg content	Total	
Local	164	16	3	19	11.59(6.7-16.5)
Cross	135	9	2	11	8.15(3.5-12.8)
Exotic	85	1	3	4	4.71(0.2-9.2)
Total	384	26	8	34	8.85(6.0-11.7)

4.2. Associated Risk Factors

Out of seventy five respondents, upon responding to egg consumption habits and preferences, 70.67% of consumers ate only cooked eggs at all times, while 29.33% of egg consumers showed preference for raw eggs. The reasons given by the respondents for eating raw eggs were presumed nutritional value (12.12%) and for medicinal purpose (87.88%). Thirty four (45.33%) respondents that practiced eating raw eggs had gastrointestinal disturbances characterized by at least one of the following symptoms: nausea and vomiting, abdominal discomfort and diarrhea, the rest 54.67% had not experienced any of the symptoms. Asked if the respondents' family members had history of consuming raw eggs, 44.00% replied that had observed family members consuming raw egg for either medicinal purpose or for presumed better nutritional value (Table 13).

The habit of washing eggs before cooking for consumption showed that 57.33% did not have habit of washing eggs and the rest 42.67% had a habit of washing eggs. The types of materials in which the eggs were stored before preparation showed that the majority 71.83% of the respondents used open containers such as carton and boxes, while only a small numbers stored eggs in refrigerators. The majority of the respondents (86%) were used this fridge storage practice to protect from breakage and the rest (14%) were to prevent egg from spoilage.

The survey on the duration of raw egg storage before preparation showed that large majority 45.07% of the respondents' stored for 7-15 days, while few respondents kept eggs for more than fifteen days. The egg consumers in Konta-Ameya town during survey were also asked to what they would do with cracked eggs and responded that 17.33% would consume it and the rest respondent's 82.67% would reject cracked egg. Out of 75 respondents, only 41.33% knew about possibility of disease transmission through raw egg consumption (Table 13).

Table 13. Questioner survey result among 75 egg sellers, farmers and consumers

No	Questionnaires	Respondents response (frequency)	
		No	**%**
1	What is the source of egg used for consumption?		
	Home made	38	50.67
	Purchased from market	22	29.33
	Both from market and own chicken	15	20.00
2	Do you consume raw egg? Yes	22	29.33
	No	53	70.67
3	Does your family consume raw egg? Yes	33	44.00
	No	42	56.00
	If yes, for what reason do they consume? For medicinal	29	87.88
	For nutritious	4	12.12
4	Have you ever had any sickness of raw egg consumption? Yes	34	45.33
	No	41	54.67
	If yes, what were the symptoms? Nausea and vomiting	11	32.35
	Abdominal discomfort	18	52.94
	Diarrhea	5	14.71
5	Do you know that disease could be transmitted by raw eggs? Yes	31	41.33
	No	44	58.67
6	Do you wash the egg before cooking to consumption? Yes	32	42.67
	No	43	57.33
7	Do you keep the eggs at home before consumption? Yes	71	94.67
	No	4	5.33
	If yes, for how many days? 1-7days	27	38.03
	7-15days	32	45.07
	15-30days and above	12	16.90
8	In what way do you keep the eggs at home?		
	Open container (carton and box)	51	71.83
	Together with crops or coffee	13	18.31
	Refrigerator	7	9.86
	Would you change after usage or reused if you used carton?		
	Changed	19	37.25
	Reused	32	62.75
9	What do you do with cracked eggs? Consumed	13	17.33
	Rejected	62	82.67
10	Which cooking method do you used? Boiling with shell	18	24.00
	Scrambling	23	30.67
	Frying/poaching	28	37.33
	Baking	6	8.00

4.3. Antimicrobial Resistance Testing

4.3.1. Levels of Antimicrobial Resistance Testing

All of the 34 *Salmonella* isolates were tested for antimicrobial susceptibility for ten different antimicrobials. All of the isolates were resistant to at least one antimicrobial agent. Of these Nalidixic acid showed maximum sensitivity and followed by Ceftriaxone and Gentamycin. Ampicillin was the most resisted, followed by Amoxicillin, Clindamycin and Tetracycline. Erythromycin, Spectinomycin and Kanamycin showed intermediate resistance (Table 14.).

Table 14. Antimicrobial susceptibility test result

NO	Antimicrobial tested	Resistant isolates		Intermediate isolates		Sensitive isolates	
		No.	%	No.	%	No.	%
1	Amoxicillin	21	61.76	9	26.47	4	11.76
2	Ampicillin	23	67.65	8	23.53	3	8.82
3	Erythromycin	6	17.65	16	47.06	12	35.29
4	Tetracycline	18	52.94	10	29.41	6	17.65
5	Kanamycin	9	26.47	13	38.24	12	35.29
6	Spectinomycin	8	23.53	15	44.12	11	32.35
7	Clindamycin	19	55.88	12	35.29	3	8.82
8	Ceftriaxone	2	5.88	11	32.35	21	61.76
9	Nalidixic acid	1	2.94	7	20.59	26	76.47
10	Gentamycin	6	17.65	8	23.53	20	58.82

4.3.2. Resistance Pattern of *Salmonella* spp. against 10 Antimicrobial Agents

All of the isolates were resistant to at least one antimicrobial agents tested, most commonly for Ampicillin, Amoxicillin, Clindamycin and Tetracycline. The number of isolates resistant to five drugs was higher followed by four drugs resistant isolates (Table 15).

Table 15. Resistance patterns exhibited by *Salmonella* isolates against 10 antimicrobial agents

***Salmonella* isolates**	**No. of isolates with same res. Pattern**	**Antimicrobial re sistance pattern**	**No.of antimicrobials developed resistance**
MR-11S, FM-23S, MR-67S, MR-80S, MR-110S, MR-136S, MR-29C, MR-40C	8	AML, CLN, SPC	3
MR-36S, FM-53S, FM-148S, FM-152S, MR-186S, MR-143C	6	AMX, AML, CLN, TET, GEN	5
FM-37S, FM-70S, MR-124S, FM-141S	4	AMX, KAN, TET	3
MR-60S, FM-190S, MR-138 C	3	AMX ,CLN, KAN	3
MR-170S, MR-187S, FM-39 C	3	AML, TET	2
MR-77C, MR-145C	2	AMX, AML, ERY, TET	4
MR-07S, FM-48S	2	AMX, AML, ERY, KAN	4
MR-29S, FM-84S	2	AMX, ERY, TET, CRO	4
FM-134S, MR-181C	2	AMX, AML, CLN	3
MR-163S	1	NA	1
MR-103S	1	TET	1

CLN: Clindamycin; ERY: Erythromycin; KAN: Kanamycin; TET: Tetracycline; AML: Ampicillin; AMX: Amoxicillin; GEN: Gentamycin; SPC: Spectinomycin; CRO: Ceftriaxone; NA: Nalidixic acid; MR: Market; FM: Farm; S: Eggshell; C: Egg content.

5. DISCUSSION

The current study was conducted on 384 chicken eggs examined to isolate *Salmonella;* the results indicated that eggshell contamination with *Salmonella* was significantly higher (6.77%) than that of egg contents (2.08%). Prevalence in the current study is higher than previous reports of 1.33% from eggshell and 0% from egg contents in Iran (Mirr-Hassen *et al.,* 2015), 2.4% from egg shell and 0.5% from egg contents from Haramaya University poultry farm (Tessema *et al.*, 2017) and 5.21% from eggs from Addis Ababa (Bayu *et al.,* 2013). This finding is in agreement with many previous reports from Ethiopia including 6.3% and 1.1% from eggshells and egg contents respectively in Mizan Teferi town, southwestern Ethiopia (Hailu *et al*., 2021), 11.5% from eggs in Kombolcha (Minte *et al*., 2011), 7.7% in eggshells from Alage (Tsegaye *et al*., 2016).

The prevalence in the current study, however, was lower than the prevalence of *Salmonella* from eggshells and egg contents 40% and 8.33% from Pakistan (Akhtar *et al*., 2010), 15.38 in eggshell and 8.33% in egg content from Mekelle (Dawit *et al*., 2016) respectively. These variations could be due to differences in chicken rearing conditions, egg collection, transportation and storage condition and storage duration which might positively or negatively affect entry and multiplication of *Salmonella* in internal egg contents or on the surface of egg shells.

Occurrence of *Salmonella* in eggs collected from local, cross, and exotic chicken breeds were 11.59%, 8.15% and 4.71% respectively. There was a statistically significant difference between eggshells of the three different breed sources ($p<0.05$). However, there was no significant difference between egg contents of different breeds. This is presumably due to unequal exposure to the risk factors as the breeds were housed in different house system. Slightly higher prevalence was observed in eggs collected from local chicken breed (11.59%) compared to those with exotic blood. This difference in prevalence might be associated with the different chicken rearing conditions and difference in susceptibility to bacteria.

Egg contamination can occur through egg contact with fecal material, insects and feed or even through transportation, storage or handling. As the hygienic standards in handling, transportation and storage improve, the *Salmonella* prevalence may lower. A number of reports of salmonellosis in eggs from developed nations, including the United Kingdom

(EFSA, 2007), where cases range from 0% to 7%, 6.1% in eggshells and 1.8% in egg contents were from retail eggs in South India (Suresh *et al*., 2006), 0.62% in the US (FSIS, 2010), 2.4% in Denmark (EFSA, 2006), and 0.4% in the Republic of Ireland (Murchie *et al*., 2007), have also been linked to the disease.

The overall source-wise the prevalence of *Salmonella* in eggs collected from poultry farms and open markets was 12(6.25%) and 22(11.46%), respectively and there was no significant difference between the two sources ($p>0.05$). There was a significant difference ($p<0.05$) in prevalence between the contents of eggs purchased from the open market and egg contents obtained from the poultry farms ($P=0.032$). In contrast to the findings of the current study, a study conducted in Kombolcha (Minte *et al*., 2011) found the prevalence of *Salmonella* in contents of egg from poultry farms (10.5%) to be significantly higher than the prevalence of *Salmonella* in egg contents from open market (3.0%) ($P<0.05$). This could be because of the *Salmonella* serotypes associated with the different chicken-rearing systems. It was known that some serotypes such as *S. Enteritidis* are known to be transmitted to eggs through the trans-ovarian route (Messens *et al.,* 2005). *Salmonella* in egg shells is a potential hazard for food-borne salmonellosis because food handlers directly handle raw eggs shell, and broken pieces of shells may come in contact with the egg content during food preparation, which could allow *Salmonella* to reach the egg contents.

In the current study Nalidixic acid (76.47%) showed maximum sensitivity and followed by Ceftriaxone (61.76%) and Gentamycin (58.82%). Ampicillin was the most resisted (67.65%), followed by Amoxicillin (61.76%) and Clindamycin (55.88%).While Erythromycin (47.06%), Spectinomycin (44.12%) and Kanamycin (38.24) most commonly turned to intermediate resistance. In this study all of the isolates were resistant to at least one antimicrobial used, and most commonly for Ampicillin, Clindamycin, Amoxicillin and Tetracycline. The high resistance patterns of *Salmonella* isolates against commonly used antibiotics are probably the result of the misuse of these antibiotics in veterinary medicine and the widespread use of antibiotics in human medicine (Castro-Vargas *et al*., 2020). Similarly, a study carried out by (Ayalu *et al*., 2011) Eastern Ethiopia, the sensitivity of the *Salmonella* isolates were 0.0% each to ampicillin and to amoxicillin 14.2% to tetracycline and 92.8% to gentamicin. Sibhat *et al*., (2011) also reported the highest level of resistance (66.7%) of *S. Newport* to streptomycin. Moreover, Zewdu and Cornelius, (2009) in Addis Ababa, Ethiopia revealed that 32 *Salmonella* isolates

were resistant to one or more of the 24 antimicrobials tested. The most common resistance was to ampicillin (59.4%), tetracycline (46.9%) and spectinomycin (40.6%) which was agreement with the present study. Jelalu *et al*., (2016), reports all *Salmonella* isolates to be resistant to erythromycin (62.5%), followed by ampicillin (37.5%), amoxicillin (37.5%), and tetracycline (25%) and also related to *Salmonella* resistance reported in Mizan Teferi Town southwest Ethiopia (Hailu *et al*., 2021) clindamycin (51.8%), ampicillin (44.4%), amoxicillin (40.7%) and spectinomycin (25.9%).

Antimicrobial resistance in the current study and previous studies such as (Hailu *et al*., 2021) appears to have developed against some of the drugs that have not been used extensively in veterinary medicine in Ethiopia. Resistance to such drugs might have originated in human medicine as well as from the environment and resistance genes might have horizontally transferred to veterinary isolates (Davies, 1994). Alternatively, as described in a relatively recent experimental study (Kohanski *et al.,* 2010), sub-lethal bactericidal antibiotic therapy (under-dosing) induces a heterologous increase in resistance to a range of other antibiotics. This increase in mutagenesis was demonstrated to be associated with an increase in the levels of free oxygen radical species that damage bacterial DNA, resulting ultimately in resistance to heterologous, sometimes to multiple antimicrobial agents that are not used for therapy.

Questionnaire results demonstrated consumers' preference for eating raw eggs in 29.33% of the respondents. The finding was similar to the reports of (Jelalu *et al.,* 2016) from eastern Ethiopia where 28% of egg consumers consumed raw chicken eggs. The reasons given by the respondents in this study for eating raw chicken eggs were for presumed nutritional values (12.12%) of raw eggs and medicinal purposes showed superiority (87.88%). The majority of the respondents (86%) used this fridge storage practice to protect from breakage and the rest 16% were to prevent egg from spoilage. Previous studies on storage, consumption patterns, and reasons for consumption of raw chicken eggs in Ethiopia (Jelalu *et al.*,2016; Minte *et al*., 2011; Hailu *et al.*,2021) demonstrated similar patterns, demonstrating widespread traditional beliefs across different cultures. These practices might result in contamination of eggs with *Salmonella* and multiplication of the pathogen in egg contents. In addition, the presence of considerable raw chicken egg consumers indicates the potential risk associated with salmonellosis from chicken eggs.

6. CONCLUSIONS AND RECOMMENDATIONS

The current study's findings indicated 6.25% and 11.46% prevalence of *Salmonella* from farms and open markets in Konta-Ameya, respectively, implying that egg products might be the cause of salmonellosis in humans. This is presumably due to unequal exposure to the risk factors as well as housed in different house system. *Salmonella* was also not very common in the contents of the eggs. The study's findings regarding antibiotic resistance suggest that chicken may be a source of multi-resistant *Salmonella* isolates, which are already a major public health risk. According to the results of the questionnaire study, the majority of egg sellers or farmers, and customers who consumed raw eggs did so because they believed it to have nutritional and medicinal value, which might have a significant detrimental impact on their health. There is also evidence of a lack of knowledge about zoonosis in the area as people in the area eat raw and cracked eggs. Thus, it is important to inform people about the dangers of eating raw chicken eggs and eggs that have cracked during storage or transit.

Based on the aforementioned findings, it is advised to take the following actions to reduce the possibility of contracting salmonellosis from eating raw eggs:

- Entire teaching or training programs for farmers, egg sellers and consumers should be offered by all accountable public health stakeholders.
- It is important to follow proper egg handling, cooking, and refrigeration procedures as well as preserve environmental cleanliness when collecting and storing eggs.
- When prescribing antibiotics, more care should be taken, and use of effective drugs and treatment of animals by professionals in order to minimize the impact of infection.
- Further investigation is needed to characterize *Salmonella* isolates to identify the species and serotype and resistant genes.

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8. APPENDICES

Appendix 1 Biochemical test result interpretation

Tests	**Positive**				**Negative**			
TSI	Slant	Butt	Gas	H_2S	Slant	Butt	Gas	H_2S
	Red	Yellow	Crack	Black	Yellow	Red	No crack	No black
Urease	red/purple				yellow/orange			
LDC	Blue				Yellow			
Indole	red ring on surface				yellow ring			
Citrate	Blue				Green			

Appendix 2 Questionnaire

1. Name ---------- Sex --------Kebele---------- Date of collection ----------
2. What is the kind of chickens? ----------
3. What is the source of the egg used for consumption?

   Home made ☐ Purchased from market ☐ Both from market and home ☐

4. Do you wash the egg before preparation for consumption? Yes ☐ No ☐
5. Do you consume raw egg? Yes ☐ No ☐

   If yes, what is the reason? Justify ----------
---

6. Does your family consume raw egg? Yes ☐ No ☐
7. If yes to Q6 for what reason do they consume raw egg?

   A) For medicinal B) for nutritious purpose C) Other (specify)

8. If yes to Q6, age group up to 5yrs ☐ 6-10yrs ☐ 11-15yrs ☐ above 15yrs ☐
9. What do you do with cracked egg? Consumed ☐ Sold ☐ Rejected ☐
10. Do you keep eggs before consumption? Yes ☐ No ☐

    If yes, for how many days do you keep?

    1 - 7days ☐ 7 – 15days ☐ 15-30days ☐ above 30days ☐

11. What is your keeping mechanism?

    At room temperature in open container (carton and box) ☐

    At room temperature together with crops and coffee ☐

☐

Refrigerator

12. Would you change after usage or reused it again if you used carton?
Changed ☐ Reused ☐

13. Have you ever had any sickness following raw egg consumption?

Yes ☐ No ☐

If yes, what were the symptoms?

Nausea and vomiting ☐ Abdominal discomfort ☐

Diarrhea ☐ Other (specify) ☐

14. Which cooking method do you used for consumption of eggs?
a) Baking b) Hard cooking c) Scrambling d) Frying/Poaching

Appendix 3 Laboratory analysis data recording format

No	**Sample Code**	**Xstics**		**Biochemical Test**								
		XLD agar	BGA agar	UA test	Citrate test	TSI agar test				MR	VP	Indole
						Slant	Butt	H_2S	GP			

KEYS: Xstics = characteristics, XLD = Xylose lysine deoxycholate agar, BGA = brilliant green agar, UA = urea agar MR = methyl Red, VP = Vagouse-prouskure test, TSI = Triple Sugar Iron Agar, GP=Gas production, H_2S = Hydrogen Sulphide Production

Appendix 4 Sample collection Format

No	Name	Sample Code	Sample Type	Source of Sample	Date of Collected	Breed	Sample collection site

Appendix 5 Procedure of isolation of Salmonella from raw chicken egg

Non-selective pre-enrichment

Swab of egg shell in 10ml and 25ml of egg content in 225ml of 10% buffered peptone water 37^{o}c, for 18-24 h,

Selective enrichment

0.1 ml culture in 10 ml of RV broth Incubation for 24h±3 at $41.5^{0}C \pm 1^{0}C$, 1ml of culture in 10ml MKTTn broth Incubation for 18-24 h, at $37°C \pm 1^{0}C$

Plating out and Identification

XLD and/or BGA plates with an inoculation loop 37°C, for 24h

Streaking on nutrient agar 37°C, 24 h

Pick five presumptive *Salmonella* colonies from each agar plate and inoculate on nutrient agar

Biochemical confirmation 37°C, 24 h

TSI and citrate	$36 \pm 1^{0}C$, 24 h
Urease,	$36 \pm 1^{0}C$, 3-24h
Indole, MP-VP	37°C, 24 h+3 minute

Expression of results

Source: ISO 6579, 2002: 2017; WHO, 2003

Appendix 6 Media used for isolation and identification of *Salmonella*

A. Buffered peptone water (BPW) (LBS. MUMBI, INDIA)

Compositions	**Gms/lit**
Tryptone	10.0g
Sodium chloride	5.0g
Disodium hydrogen phosphate $12H_2O$	9.0g
Potassium dihydrogen phosphate	1.5g
Final pH (at 25°C	7.0 ± 0.2

Direction: Suspend 20.07gms in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense in tubes or flasks required amount. Sterilize by autoclaving at 121°C /15 Ibs pressure for 15 minutes.

B. Rappaport-Vassiliadis Medium (RV) (LAB, M 880-500gm, India)

Compositions	**Gms/lit**
Soya bean meal	4.5g
Sodium chloride	7.2g
Monopotassium phosphate	1.44g
Dipotassium hydrogen phosphate	0.18g
Magnesium chloride	36.0g
Malachite green	0.036g
Final pH (at 25 °C)	5.2 ± 0.2

Direction: Suspend 49.17grams of powder and dispense into 1liter of distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired in to tubes and Sterilize by autoclaving at 115^{o}C for 15 minutes.

C. Muller-Kauffmann Tetrathionate Novobiocin broth base (MKTTn) (LAB M14961-500gm, India)

Compositions	**Gms/lit**
Peptone	4.30g
Casein enzyme hydrolysate	8.60g
Ox bile	4.75g

Sodium chloride	2.60g
Calcium carbonates	38.7g
Sodium thiosulphate, $5H_2O$	47.8g
Briliiant green	0.0095g
Final pH (at 25°C)	8.2 ± 0.2

Direction: Suspend 89.42gms in 1000ml of distilled water. Heat the medium just to boiling. Do not autoclave. Cool to 45-50°C and just before use aseptically add 20ml of iodine solution (20gm iodine and 25gm potassium iodide in 100ml sterile distilled water). Along with rehydrated contents of one vial of MKTTn supplements (FD-203). Mix well before dispensing in the sterile tubes to disperse calcium carbonate uniformly.

D. Xylose Lysine Deoxycholate Medium (XLD) agar (MIL-CHM-081(CM0469), England

Compositions	**Gms/lit**
Yeast extracts	3.0g
L-Lysine HCL	5.0g
Xylose	3.75g
Lactose	7.50g
Sucrose	7.50g
Sodium deoxycholate	1.0g
Sodium chloride	5.0g
Sodium thiosulfate	6.8g
Ferric Ammonium citrate	0.8g
Phenol red	0.08g
Agar	12.5g
Final pH (at 25 °C)	7.4± 0.2

Direction: Suspend 53gm in 1liter of distilled water. Heat with frequent agitation until the medium boils. Do not over heat. Transfer immediately to a water bath at 50°C pour in to plates as soon as the medium has cooled. It is important to avoid preparing large volume which will cause prolonged heating.

E. Brilliant Green Agar (BGA) (ISO-CAT, Canada)

Compositions	**Gms/lit**
Bacteriological Agar	20.0g
Brilliant green	0.0125g
Lactose mono hydrate	10.0g
Peptone	10.0g
Phenol red	0.08g
Sodium chloride	5.0g
Sucrose	10.0g
Yeast extracts	3.0g
Final PH (at 25 °C)	6.9± 0.2

Direction: Suspend 58.1gms of medium in 1liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boiling for one minute until complete dissolution. Sterilize in autoclave at 121°C for 15 minutes. Cool to 45-50°C. Mix well and dispense in to plates. If necessary allow to dry about for approximately 2hrs with covers partially removed.

F. Triple Sugar Iron (TSI) Agar (OXOID, India)

Compositions	**Gms/lit**
HMC peptone	20.0g
HM peptone B#	3.0g
Yeast extracts	3.0g
Lactose monohydrate	10.0g
Sucrose	10.0g
Glucose monohydrate	1.0g
Ferric ammonium citrate	0.3g
Sodium chloride	5.0g
Sodium thiosulphate	0.3g
Phenol red	0.025g
Agar	12.0g
PH (at 25 °C) after sterilization	7.4± 0.2

Direction: Suspend 64.02gms in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Cool to 45-50°C. Mix well and distribute into test tubes. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes or as per validated cycle. Allow the medium to set in sloped from with a butt about 1 inch long.

G. Urea agar base Christensen (M112I-500gm, India)

Compositions	**Gms/lit**
Peptone	1.0g
Dextrose (Glucose)	1.0g
Sodium chloride	5.0g
Disodium phosphate	1.2g
Potassium dihydrogen phosphate	2.0g
Phenol red	0.012g
Agar	15.0g
Final PH (at 25 °C)	6.8 ± 0.2

Direction: Suspend 24.01gms in 950 ml of distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving 115°C for 20 minutes. Cool to 45-50°C and aseptically add 50ml of sterile 40% Urea solution (FD-048) and Mix well, Distribute in to sterile tubes and allow to set in the slanting position. Do not over heat or reheat the medium as urea decomposes very easily.

H. Simmons citrate agar (M099-500gm, India)

Compositions	**Gms/lit**
Magnesium sulphate	0.2g
Ammonium dihydrogen phosphate	1.0g
Dipotassium hydrogen phosphate	1.0g
Sodium Citrate	2.0g
Sodium chloride	5.0g
Bromothymol blue	0.08g
Agar	15.0g
Final PH (at 25 °C)	6.8 ±0.2

Direction: Suspend 24.28gms in 1000 ml of distilled water. Heat to boiling to dissolve the medium completely. Cool to 45-50°C and Mix well and distribute in tubes or flasks as desired. Sterilize by autoclaving 121°C for 15 minutes.

Precaution: Before using water insure PH of water is 6.5-7.0. Initial color of medium may deviate from expected color if the above precaution is ignored.

I. MR-VP medium (Glucose phosphate broth) (M070-500gm, HimediaLab.pvt. Ltd India)

Compositions	**Gms/lit**
Buffered Peptone	7.0g
Dextrose	5.0g
Dipotassium phosphate	5.0g
Final PH (at 25 °C)	6.9± 0.2

Direction: Suspend 17.0gm in 1000ml distilled water. Heat if necessary to boiling to dissolve the medium completely. Distribute in test tubes in 10ml amounts and Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes.

J. Tryptone broth (for Indole production) (500gm, New Mumbi, India)

Compositions	**Gms/lit**
Pancreatic digest of casein	10.0g
Sodium chloride	5.0g
Final PH (at 25 ^{0}c)	7.5± 0.2

Direction: Add 15.0gm powder in to 1000ml distilled water and mix thoroughly. Gently heat and bring to boiling. Distribute in test tubes in 10ml amounts and Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes.

K. Motility test medium (AL1718-00500gm, Alpha chemika, India)

Compositions	**Gms/lit**
Tryptose	10.0g
Sodium chloride	5.0g
Agar	5.0g
Final PH (at 25 °C) after sterilization	7.2± 0.2

Direction: Suspend 20.0gms in 1000ml purified/distilled water. Heat to boiling to dissolve the medium completely. Dispense in test tubes and Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes. Allow tubed medium to cool in an upright position.

L. Nutrient agar (NA) (M001A-500gm, India)

Compositions	**Gms/lit**
Peptone	5.0g
HM Peptone B#	1.0g
Yeast extracts	2.0g
Sodium chloride	5.0g
Agar	15.0g
Final PH (at 25 °C)	7.4± 0.2

Direction: Suspend 28.0gm in 1000ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes. Cool to 45-50 °C. Mix well and pour in to sterile Petri plates.

M. Tryptone Soya broth (M011-500gm, HimediaLab.pvt. Ltd, India) or bacterial suspension

Compositions	**Gms/lit**
Tryptose	17.0g
Soya peptone	3.0g
Sodium chloride	5.0g
Dipotassium hydrogen phosphate	2.5g
Dextrose (Glucose)	2.5g
Final PH (at 25 ^{0}c)	7.3± 0.2

Direction: Suspend 30.0gms in 1000ml purified/distilled water. Heat if necessary to dissolve the medium completely. Mix well and dispense in tubes or flasks as desired. Sterilize by autoclaving at 15 Ibs pressure (121°C) for 15 minutes. Allow tubed medium to cool in an upright position.

Note: If any fibers are observed in the solution it is recommended to filter the solution through a 0.22 micron filter to eliminate the possibility of presence of fibers.

N. Mueller Hinton Agar (M173-500gm, India)

Compositions	**Gms/lit**
HM infusion Beef	300.0g
Casein hydrolysate	17.5g
Starch	1.5g
Agar	17.0g
PH at 25 °C	7.3 ± 0.1

Direction: Suspend 38g in 1 liter of purified/distilled water. Heating to boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50°C. Mix well and pour in to sterile Petri plates.

Appendix 7. Some of the pictures taken during the processing (analysis) or in isolation

Appendix 8. Some pictures showing biochemical test and result interpretation

Appendix 9. Some pictures showing antimicrobial susceptibility test result