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Occurrence and antimicrobial resistance of *Salmonella spp., Staphylococcus aureus and Escherichia coli* O157:H7 from chicken eggs in retail market & small scale poultry production center in Addis Ketema sub city, Addis Ababa, Ethiopia.

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This is to certify that the thesis prepared by Demis Alamrew, entitled:

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Abbreviations

AAU	Addis Ababa University
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
BA	Blood agar
C+veSC	Coagulase Positive Staphylococci Count
CC	Coliforms Count
CDC	Centers for Disease Control and Prevention
CDDEP	Center for disease dynamics, economic and policy
CFC	Colony forming unit
CLSI	National Committee for Clinical Laboratory Standard
DRERC	Department Research and Ethical Review Committee
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
Mac	MacConkey agar
NCCLS	National Committee for Clinical Laboratory Standard
PCR	Polymerase chain reaction
RV	Rappaport-Vassiliadis broth
SFD	Staphylococcal food-borne disease
SMac	Sorbitol MacConkey agar
SOP	Standard Operating Procedures
SS agar	*Salmonella Shigela* agar
TSA	Tripticas soya agar
TTC	Thrombotic thrombocytopenic purpura
USA	United States of America

USDA	United States Department of Agriculture
VRBA	Violet red bile agar
WHO	World Health Organization
XLD Agar	Xylose lysine deoxycholate agar

Abstract

Background: Chicken egg is considered as a nutritionally complete food and an excellent source of protein. While highly nutritious, eggs are also one of the leading causes of food poisoning and food borne illness in the world. In poultry industries antibiotics are used as a method of preventing, growth promoters and treating illness within the flocks. This contributes to the development of bacterial resistance to antibiotics.

Objective: To assess the occurrence and antimicrobial resistance pattern of *Salmonella spp*., *Staphylococcus aureus* and *Escherichia Coli* O157:H7 from chicken eggs in Addis Ketema sub city, Addis Ababa, Ethiopia.

Methods: A total of 380 eggs (76 composite sample) were collected from small scale producers and retail markets in the study area over a period of 6-month. The eggs were screened for *Salmonella spp*., *S. aureus* and *E. coli* O157:H7 following standard procedures. Isolated bacteria were subjected to antimicrobial susceptibility test. Frequencies were used to assess the magnitude of microorganisms on shells and in egg contents. Independent and dependent variables were associated by kruskal Wallis tests using SPSS version 23.

Results: About 14(9.2 %), 9(5.9 %) and 18(11.8 %) of the 76 composite samples were positive for *Salmonella spp.*, *S. aureus* and *E. coli* O157:H7 respectively. The shells showed significantly higher ($p<0.05$) level of contaminations than the contents for both *Salmonella spp.* and *E. coli* O157:H7 microorganisms. On the other hand, *S. aureus* highly contaminant on shell than content eggs even statically not significant. *Salmonella spp*. showed the highest resistance patterns against ampicillin (64.3 %), cefoxitin (35.7 %) and ciprofloxacin (35.7 %). *E. coli* O157:H7 showed highest resistance against ampicillin (72.3 %), followed by cefoxitin (55.7 %) and tetracycline (27.8 %). In case of *S. aureus*, the greatest number of isolates showed resistance to tetracycline (100 %) oxacillin (88.9 %), penicillin (77.8 %), erythromycin (44.4 %) and clindamycin (44.4 %).

Conclusion: From the result of the study, the eggs marketed for human consumption in Addis ketema sub city, Addis Ababa is contaminated with *Salmonella spp*., *S. aureus* and *E. coli* O157:H7 resistance to antimicrobial agents commonly used in veterinary and human practice. Therefore, the egg should be taken with caution and the public should be educated on the dangers associated with consumption of raw and under cooked eggs and egg products.

Key Word: Eggs, Bacterial quality and safety, *Salmonella spp., E. coli* O157:H7, *S. aureus*, AST

1 Introduction

1.1 Background

The chicken egg is the end products of a complicated series of processes formed gradually over a period of approximately 25hrs and many organs participate for the formation of different part of the egg. When fully formed, the average weight of a large egg is 57g and 38g of this weight (66 %) is made up of water; It consists of approximately 10 % shell, 58 % white and 32 % yolk (1). The first recorded consumption of eggs produced by domestic fowl dates back to approximately 1400 B.C. in both Egypt and China (History of Egg Production) (2). Chickens used as a source of food during the Roman Empire and its significant role in religious holidays are credited with the development of breeds, especially for egg production (3).

Eggs have represented an important part of the human diet, because it is inexpensive food and nutrient rich, containing proteins, minerals, fats, vitamin B_{12}, Riboflavin, choline and more needed for a good health (2)**.** Due to the protein content, the United States Department of Agriculture (USDA) categorized eggs as meats within the Food Guide Pyramid (4). There is also scientific evidence that eggs contain biologically active compounds with antimicrobial, antioxidant, anti-cancer properties which have important implications for the pathophysiology of numerous chronic diseases and immune responses to acute injury (3, 5). While eggs are highly nutritious for humans, it is also nutritious or creates an appropriate environment for the development of microorganisms like microbes, including pathogenic bacteria (5, 6).

Freshly laid eggs are generally sterile however, in a relatively short period of time after laying, eggs become contaminated with different types of microorganisms due to its exposure to environmental conditions such as faces, soil, water, nesting material, hands and insects (7). Furthermore, these microorganisms may contaminate the egg contents which may lead to spoilage and pathogen transmission (8). There are two possible routes of bacterial contamination of eggs, vertically or horizontally (9). In the vertical transmission the egg content is directly contaminated as a result of bacterial infection of the reproductive organs of infected hens, i.e. ovaries or oviduct tissue (10). In the horizontal transmission contamination occur when eggs are subsequently exposed to a contaminated environment and micro-organisms penetrate through the egg shell pores (1,11). The major contamination of the egg is due to horizontal transmission (12). Eggs normally contaminated with both gram negative bacteria and a few gram-positive

organisms. Gram positive bacteria, probably because of their tolerance of dry conditions, are the dominated micro flora of the eggshell, whereas gram-negative bacteria are the principal contaminants of rotten eggs because they are best equipped to overcome the antimicrobial defenses of the egg content. *Salmonella spp.*, *Listeria monocytogenes*, *E. coli*, *Campylobacter strains* and *S. aureus* are some of the common bacterial strain that causes food borne disease in related to egg contamination (13).

In spite of these bacterial contamination, egg is equipped with physical and chemical defenses against microbial contamination (2). Physical resistance of egg to bacterial contamination is provided by cuticle, egg shell, and shell membrane (9). Cuticle is the primary barrier which limits the movement of particles, water and bacteria through the shell pores. In addition to physical protection, its chemical composition like proteins and lipid's antimicrobial activity plays an important role in limiting bacterial contamination (14). However, this defense is not perfect because small percentage of eggs are laid without cuticle and the first few minutes after lay it is an ineffective barrier to bacterial invasion until it become hardens (2). The cuticle thickness along the egg shell surface was quite variable, even being completely absent in some regions of the egg shell (15). The egg shell is another very important visible physical barrier. The egg shell, a thin mineral structure, protects the egg contents against mechanical impact, dehydration, and microorganism contamination (10). In addition to physical defense eggshell also participate in biological defense mechanism (14).

The third effective mechanical barrier is egg shell membranes. There are two shell membranes (inner shell membrane and outer shell membrane) that are held closely together except at the blunt end of the egg where the air cell is located (16). In relation to bacterial penetration, the shell membranes act as a filter, being more impenetrable to bacteria than the shell (17). In addition to these physical protective barriers albumen also contributes to the mechanical protection against microorganisms (18). In mechanical defense, viscosity hampers the movement of bacteria that invade the shell membranes towards the yolk and the combined action of the chalazae and aluminous sac of fresh eggs contributes to the central location of the yolk, thus maintaining it at the greatest distance from the shell membranes (1,2). If microorganisms successfully penetrate the physical barriers, they will face a number of chemical barriers (19). The chemical defense consists of proteins that exhibit antimicrobial activity. A number of proteins have been identified in chicken egg; however, all of them have reported to be not

involved in antimicrobial defense mechanism (20). The antimicrobial egg proteins either cause direct degradation of microbial components or chelation of vitamins, minerals essential for microbial growth and/or inhibition of bacterial proteases involved in the invasion of pathogens (10).

Conalbumin (Ovotransferrin) is an iron-binding agent that quencher with iron and inhibit bacterial multiplication by depriving iron necessary for their growth (19). Lysozyme causes hydrolysis of β-1, 4-glycosidic bonds in peptidoglycans of bacterial cell well so it makes bacterial growth difficult in the egg. It has the highest lytic activity against gram positive bacteria than gram negative bacteria (21). Avidin is another biological active substance with antimicrobial properties in albumin which inhibits the growth of bacteria by binds with biotin and surface receptor of various bacteria. Ovoinhibitor inactivates several proteases, ovoflavoprotein chelates riboflavin and Ovomucoid inhibits trypsin; Alkaline PH of albumin is also hinder the growth of most bacteria in egg content and prominent the chelating potential of ovotransferrin (10).

Chicken table eggs are the most critical food vehicles of pathogenic microorganisms participating in the etiology of food borne diseases in humans, which are among the most widespread global public health problems of recent time. The consumption of infected eggs cause different food-borne diseases which can be treated only by the use of chemotherapeutic agents (22). *Salmonella* is a gram negative rod, facultative, motile and none spore former that are found in different hosts and environments that can cause human illness such as enteric fever, gastroenteritis, septicemia and salmonellosis (23). Salmonellosis is an important zoonosis and one of the most common and widely distributed food-borne diseases. It is transmitted from healthy carrier animals to humans through contaminated food (24); its outbreak in human predominately linked to the consumption of contaminated eggs and raw egg products (11). The predominant serotypes of salmonella responsible for non-typhoidal salmonellosis are *Salmonella enterica* serotype Enteritidis and Typhimurium (25). It is interesting to note that if these microorganisms are present in foods, there is no taste, smell or appearance difference and no clinical signs in the adult hen and cannot be observed by the naked eye in the shell egg (26). *Escherichia coli* are a gram negative, none spore forming rod bacterium that infect humans through consumption of contaminated food and water (27). Most strain of *E. coli* is nonpathogenic and/or opportunistic but some strains are pathogenic which are distinguished

from normal flora by their possession of virulence factors such as exotoxins, enterotoxin, and resistance to the lytic action of the host complement and antibiotics (28). The common *E. coli* strains that predominantly spread through food chain and causes food born disease is *E. coli* O157:H7 (29). *E. coli* O157:H7 is an emerging public health concern in most countries of the world (30). The first reported isolation of *E. coli* O157:H7 from cattle with colibacillosis was in Argentina in 1977. The first confirmed isolation of *E. coli* O157:H7 was in 1975 from a California woman with bloody diarrhea (29). This was first identified in 1982 as a human pathogen in the United States of America and cattle have been identified as a major source of *E. coli* O157:H7 infection of human (23, 24). It is a major pathogen of commercial poultry causing colibacillosis with the manifestations of pericarditis, septicemia, and death of the birds (32). Chicken and hen eggs have been considered as vehicles of transmission of *E. coli* O157:H7 (31). This pathogenic microorganism causes hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC) and thrombotic thrombocytopenic purpura (TTC) (26, 28).

The third pathogen I would like to consider is *Staphylococcus aureus.* It is gram positive cocci that cause food poisoning disease worldwide resulting from the contamination of food by preformed *S. aureus* enterotoxin (35). Among all foodborne diseases in the world, Staphylococcal food poisoning is ranked as the third (36). Improper food handling practices in the retail industry account for the majority of Staphylococcal food-borne disease (SFD) outbreaks (37). *S. aureus* is an important pathogen due to combination of "toxin-mediated virulence, invasiveness, antibiotic resistance", grow in a wide range of temperatures (7^0 to 48.5^0c; optimum 30^0c to 37^0c), pH (4.2 to 9.3; optimum 7 to 7.5), and sodium chloride concentration up to 15 % NaCl and its ability to survive in potentially dry and stressful environment (38). It was the first bacteria that develop penicillin resistance and has since developed resistance to a wide range of antibiotics. Antibiotic resistance *S. aureus* strains may be emerged from animal when bacteria such as enterococci from animal transfer resistance gene to human pathogenic strains (39).

Antibiotics are natural, synthetic, or semi-synthetic substances which interfere growth of or kill microorganisms, specifically bacteria, and are used to treat or prevent infections in humans and animals (WHO, 2015a). Antimicrobial resistance (AMR) is the ability of microorganisms (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarial) from working against it (2).

Antimicrobial resistance is an increasingly global problem, and emerging antimicrobial resistance has become a public health issue worldwide (40). It has now been escalated by major world health organizations to one of the top health challenges facing the 21st century (41). A variety of foods and environmental sources harbor bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine (42). Since 1946, antimicrobials have been used by the poultry industry to enhance growth, feed efficiency and to reduce bacterial diseases (2). The use of antimicrobial in poultry industry was first demonstrated in the USA and later, most of the poultry industry has led to the use of large amounts of antimicrobials in food animals. The use of antibiotics in poultry industry, particularly for growth promotes and prevention and treatment of disease are a contributor factor in emergence of antibiotic resistance bacteria in human (43). According to a study which was conducted in India by US based center for disease dynamics, economics and policy (CDDEP) indicate that consumption of chicken and eggs could exposed a man for resistance of antibiotics (44).

World Health Organization (WHO) organize workshops and meeting because of indiscriminate use of antimicrobials both in human and veterinary medicine were the sources of increased antimicrobial resistance bacteria population with resulting adverse consequences for the prevention and treatment of diseases in humans and animals (43). Gram negative bacteria are generally more resistance to antibiotics than gram positive bacteria because gram negative bacteria have a unique outer membrane that prevents penetration of drugs and antibiotics to the cell (45). Different types of antibiotic-resistant bacteria have increasingly been identified in food animals, products, and feeds as well as in humans. Such antibiotic-resistant bacteria are either indicator or commensal, foodborne or emergent organisms (46).

1.2 Statement of the problem

Foodborne illness is one of the most serious health problems affecting public health and development in the worldwide (47). Food of animal origin, especially poultry, poultry products and raw eggs are implicated as the most common cause of food-borne infection (48). In the USA, it was estimated that the annual incidence of foodborne illness was 48 million cases and the economic burden estimate ranges from US $51.0–77.7 billion (49). In Canada, annual estimates of foodborne illness range from 3.1–5.0 million cases and in Australia the incidence is estimated at 5.4 million cases costing AUD$1.2 billion annually (50).

Salmonella is an important foodborne pathogen worldwide. Outbreaks of *Salmonella* serotype Enteritidis have been repeatedly associated with the consumption of raw and undercooked eggs. 93.8 million Human cases of gastroenteritis and 155 000 deaths occur due to *Salmonella* infection around the world each year (51). In the USA alone, it was estimated that around 1.4 million illnesses and 600 deaths caused by salmonellosis annually, with the most common serotypes identified as *S.* typhimurium and *S.* Enteritidis (50) and its socio-economic losses of about USD1.1 billion (52).

Egg-associated salmonellosis is one of the main causes of foodborne illnesses worldwide. Typically contaminated eggs and egg related products are primary vehicles for human salmonellosis(53). About 80 % of *S.* Enteritidis infection outbreaks from 1985 through 1999 were associated with contaminated eggs (54). In 2009, there were a total of 6.2 million cases in the European Union and 44 % of confirmed foodborne bacterial infections in the USA were caused by *Salmonella* (43, 44). In Australia in 2011, 45 % of all salmonellosis outbreaks were linked to eggs or egg-related products. In the U.K., a large outbreak of *Salmonella* typhimurium definitive type 49 was linked to eggs with raw-egg mayonnaise identified as the likely cause of infection (50). In Spain in the period 1993 and 2002, 9364 food-borne outbreaks were reported, 4944 (52 %) of which were caused by *Salmonella* and 3546 (37.8%) of which were associated with egg products (55). In 2010, an outbreak of salmonellosis from egg shells affected more than 2,000 people in at least five states of USA (CDC 2010b, 1).

Roseline Y. et al. reported that 59 % of bacteremia in children were caused by non typhoidal *Salmonella* most commonly acquired from food sources among that *Salmonella* typhimurium and *Salmonella* Enteritidies were responsible for 82 % of the case , similarly one retrospective

study done in one hospital of democratic republic of Congo from 2002 -2006 non typhoidal *Salmonella* caused by contaminated poultry meat and eggs were reported to cause 120 cases of childhood bacteremia per 100000 persons / year (56). A study done in Harare, Zimbabwe point out chicken and egg consumption was an important cause of *Salmonella* Entritidis in human after they were observed a similar distribution of *Salmonella* Entritidis in chicken, eggs and human (24).

Staphylococcal food poisoning is the third common foodborne diseases in the world and is of major concern in public health programs worldwide (36)**.** A variety of foods can support the growth of *S. aureus* including chicken eggs (57)**.** Foods high in starch and protein are believed to favour Staphylococcal enterotoxins (SEs) production (58)**.** Nearly, 50 % of *S. aureus* produce enterotoxins which create food poisoning in consumers (59)**.** Animal originating *Staphylococcus* strains can potentially be harmful to humans (60). Eggs are the potential source of transmitting antibiotic-resistant *Staphylococcus* strains to human causing food-borne infection (36)**.** *S. aureus* is a significant cause of FBD, causing an estimated 241,000 illnesses per year in the United States (61)**.** In Japan *Staphylococcal* food poisoning outbreak were happen in 21 dam construction workers due to ingestion of boxed lunches that contain scrambled egg prepared in company cafeteria (38).

E. coli O157:H7 is an emerging public health concern in most countries of the world (30). Due to low infectious dose and life-threatening complications of *E. coli* O157:H7 the organism has emerged as an important zoonotic agent, causing serious public health problems. Most *E. coli* O157:H7 outbreaks are food or water-related (62). The occurrence of *E. coli* O157 and *E. coli* O157:H7 multiple antibiotic resistant profiles may show a risk for public health and food safety as well as animal health and production (30)**.**

Since chicken eggs are implicated as the most common cause of foodborne infections and very few works has been done in the area regarding microbial quality of chicken eggs. Early and accurate detection of pathogenic bacteria is important to undertake appropriate control measure. Inappropriate use of antibiotics in animal food production for growth promotion and routine disease prevention has necessitated the development of drug resistance. Therefore, periodic surveillance of antibiotic susceptibility profile of pathogenic bacteria's in poultry farms is important with regard to management and treatment of infection.

1.3 Significance of the study

Microbial food safety is an increasing public health concern worldwide to prevent food borne illness. Microbial contamination of egg should be controlled in order to obtain eggs with quality for human consumption. The contamination of the egg contents with microorganisms may lead to transmission of pathogens inducing cases of food-borne infection to consumers constituting a public health hazards. Owing to the continuous consumer demands for eggs, it is extremely necessary not only to increase egg production but also to safeguard consumers against health hazards, so the aim of this study to investigate bacteriological quality and safety of chicken table eggs is important to egg producers to protect public health and to satisfy the expectation of consumers.

The data of the study changes consumer and/or public perception of a "good egg" from shell cleanliness and physical properties to that of microbial integrity. The data will be helpful in creating awareness and discouraging general public for the consumption of raw or half cooked eggs to reduce infection in consumers. Especially pregnant women, babies, older adults and people with weakened immune systems should use egg and egg products with caution.

Due to current consumer concern for food safety, our study may be beneficial for small scale poultry producer to examine microbial integrity of table eggs as a screening tool to enhance table egg safety and quality and to implement quality assurance program to ensure the safety of the nation's egg supply and provide consumers with a safe and wholesome product.

The use and misuse of antimicrobial agents in poultry industries contribute to the development of antibiotic resistance; In Ethiopia, the costs of antibiotic resistance in human medicine, eg. the need to use more expensive antibiotics, multiple courses of antibiotics, increased length hospital stay, increased morbidity and mortality are largely born by government and patients. Therefore, this study will be used for governments to implement monitoring systems based on the resistance pattern of strains isolated from chicken eggs and to implement policies to regulate egg producers for treating and controlling microbial infections in eggs and proper use of antimicrobial agents in poultry industries.

2 Literature review

2.1 Magnitude of bacterial isolates in chicken eggs

A nationwide survey conducted in Uruguay to assess the prevalence of *Salmonella* in Poultry and Eggs demonstrating a prevalence of at least 1 in every 214 eggs (25). A survey of the microbial quality of table eggs sold in Trinidad revealed both egg shells and egg contents were positive for *Salmonella*, *Escherichia coli* and *Campylobacter* (65). Van Duijkeren et al. (2002) indicate *Salmonella* Enteritidis being the most predominant serotype in poultry because the percentage of isolates belonging to the serotype *Salmonella* Enteritidis increased in the Netherlands from about 5.5 % in 1986 till 15 % in 1992 and about 20 % in 2000 (66).

Mahdavi M. et al. (2012) found several pathogenic microorganisms from chicken egg shells and contents in Iran. The well-known enteric pathogens particularly *E. coli*, *S. aureus*, *Campylobacter spp*. and *Pseudomonas aeruginosa* except *Salmonella* and *Listeria* have been isolated from table egg shell and their contents (11). Nazer A. et al. (2006) also revealed that hen's egg in Iran are often contaminated with different bacteria, *Staphylococcus spp*., *Klebsiella spp*., and *Escherichia coli* were the most frequently isolated organisms (63).

A study which was conducted in Taif city (Saudi Arabia) indicates that both gram-positive and gram-negative bacteria were isolated with different rate of contamination. *Salmonella spp*., *E. coli* O157:H7, *Listeria* and *Campylobacter* were the isolated bacteria (9). Amalf A. et al. (2015) also done analysis for bacterial contamination on their shells and internal contents and report *E. coli*, *Enterobacter spp*., *Klebsiella spp*., and *S. aureus* were the isolated bacterial species (16). Mahfuzul I. et al. (2018) reported that *Escherichia coli* (34.64%), Coagulase positive *Staphylococcus* (24.29 %), *Salmonella spp*. (20.71 %) were the major contaminants of egg (67). Samah E. et al. (2015) revealed that *E. coli* and Coagulase Positive Staphylococci were detected from egg sample but *Salmonella* was not detected (68). Another study done by Jannatul F. et al. (2016) to isolate and identify pathogenic gram-positive bacteria from egg shell of hen indicates different bacteria like *Staphylococcus spp*. *Streptococcus spp. and Bacillus spp.* were detected from the egg sample (69).

Saad M. F et.al (2015) examined a leaking chicken eggs to assess the microbial quality of egg in Egypt reflects different types of pathogenic bacterial species except *Salmonellae spp*. were isolated. The biochemical identification of coliform organism in the examined sample indicates

that *E. coli* (28.58 %) were the most common isolated bacteria (22). Another study in Bangladesh also states that *Escherichia coli* was the highest percentage of bacterial pathogens present in egg shell and content in addition *Staphylococcus spp*. and *Salmonella serovars* were isolated (70). A study done in Iran also shows *E.coli* was the most prevalent bacteria in egg sample however; *Salmonella spp.* was not detected in all egg samples analyzed in food microbiology laboratory (47).

A study done in Mekele and kombolcha revealed that chicken table egg was contaminated with *Salmonella* (54, 59). Ewonetu K. et al. (2015) in eastern Ethiopia states egg shell and contents were contaminated with different microbial organisms including bacteria (73).

2.2 Comparison of bacterial contamination rate in egg shell and egg content

Different study examined samples of egg shells and egg content indicate that the shell samples have the highest microbial load while the internal content have the lowest load of bacterial count**.** Arathy Sabarinath et al. (2009) evaluate the microbial quality of eggs that are sold for human consumption in Grenada and indicate the major contaminants are gram negative bacteria belonging to the family *Enterobacteriaceae*, though *Staphylococcus spp*., *Streptococcus sp*., *Micrococcus spp*., and *Bacillus spp*. have also been isolated in small percentage (23)**.** A study conducted in the Tamale municipality in the Northern Region of Ghana to assess the Microbial quality of table eggs sold on selected markets indicates that all the egg samples have microbes of different genera on the shell; whereas, the egg contents were contaminated with a few microbial genera (74).

A study conducted in Butaleja, eastern Uganda found that bacterial contamination of egg was higher in egg shell than egg content. The microbial analysis of the study revealed that many medically important bacteria's were found on the egg shell such as *Escherichia coli* (19 %), *Staphylococcus aureus* (18 %), *Proteus* (2 %), *Pseudomonas* (9 %), but only *Pseudomonas* and *staphylococcus* spp were detected in egg content. *Salmonella ssp*. was not isolated from both egg shell and content in this study (75).

Another study conducted in Abidjan, Ivory Coast also revealed that microorganisms were not detected from the eatable part of sampled eggs, but various kinds of microorganisms such as *E. coli, Staphylococcus, Enterobacteriaceae* and anaerobic sulphite-reducing bacteria were detected from the shall (76). In Nigeria all egg shell samples collected from 16 randomly selected retail

outlets were contaminated with microbes of different genera however, only 59.4% of the samples content was contaminated with *Esherichia coli*, *Staphylococcus spp.*, *Streptococcus*, *Bacillus*, *Salmonella*, *Klebshella*, *Proteus* and *Shigella* (77).

According to a study done by Nahashon SN et.al (2016) to investigate the prevalence of *Enterobacteriaceae* in eggs shell and content from small poultry farms and farmers' markets using a total of 504 eggs was displayed as *Escherichia coli* (11.9 and 5.2 %), *Enterobacter* (9.1 and 7.9 %), and *Serratia* (11.5 and 4.8%) were positive for Shell eggs and egg contents respectively, 3.6% *Salmonella spp*. was isolated only from the egg shells (78).

Another study conducted by ashish kr et.al (2017) examined a total of 132 egg samples indicts that the risk of contamination by microorganisms was higher on egg shell than that of albumin and yolk. 28.7 % of examined samples were contaminated by microorganisms; from the total prevalence 24.2 % were isolated from eggshell, 3 % from albumin and 1.5% from yolk (45).

A study conducted in Haramaya University Poultry Farm by Tessema K, et al. Indicate that 2.9 % of egg samples were positive for *Salmonella spp*., 2.4 % *salmonella spp* was detected from egg shall and 0.5 % from egg content (39). A study conducted in Mekele also indicates that the prevalence of *Salmonella* in egg shells was higher than egg contents. It showed that 15.38 % and 8.33% were the prevalence of *Salmonella* in egg shell and content respectively (71).

2.3 Antimicrobial susceptibility pattern of isolated bacteria

A reviewed data on AMR in 12 poultry pathogen shows pathogenic *Escherichia coli* considerably higher levels of AMR compared with *Salmonella spp*. with prevalence of resistance over >80% for ampicillin, amoxicillin, tetracycline across studies (40).

Dadras, H. et.al (2015) shows *Bacillus spp*., *E. coli, Klebsiella spp., Staphylococcus spp.* and *Streptococcus spp*. isolated from eggs were resistant to enrofloxacin, erythromycin, and furazolidone whereas they were sensitive to chloramphenicol (7). All *Staphylococcus* strains isolated from chicken eggs shells in Abidjan, Ivory Coast were resistant to tested antibiotics such as oxacillin, quinolones, amoxicillin and amoxicillin + clavulanic acid but highly sensitivity to carbapenems and monobactams. *E. coli* strains were sensitive to carbapenem, cephalosporins, monobactam and quinolones except amoxicillin and amoxicillin+clavulanique acid (76) . According to Nahashon SN et.al (2016) study *Enterobacteriaceae* in shell eggs were resistant to antimicrobial agents, erythromycin was highly resistance antimicrobial agent followed by

ampicillin and tetracycline (78). Another study conducted in Jaipur, India also indicates 73.3 % of the isolated bacteria were found to be multi drug resistant against three or more antibiotics tested. The highest resistance rates were recorded against cefixime (86.66 %), amoxicillin (80 %) and amoxyclave (73.33 %) (45).

A study conducted in Haramaya University Poultry Farm showed that 72.7% of the isolated *Salmonella spp*. was resistant to one or more of the tested antimicrobials. The most common resistance was observed to tetracycline (72.7%), ampicillin (72.7%) and amoxicillin (63.6%). However, chloramphenicol were sensitive against most of the *Salmonella* isolates (39).

3 Objective

3.1 General objective

To assess the occurrence, association and antimicrobial resistance of *Salmonella spp*., *Staphylococcus aureus* and *Escherichia coli* O157:H7 from chicken egg in retail market & small scale poultry production in Addis Ketema sub city, Addis Ababa, Ethiopia.

3.2 Specific objectives

- To assess the occurrence of *Salmonella spp*., *Staphylococcus aureus* and *Escherichia Coli* O157:H7 from chicken eggs in the retails markets and small scale poultry production system.
- To determine the antibiotic susceptibility pattern of isolated bacteria from the examined sample.
- To assess the association between various factors and occurrence of *Salmonella spp*., *Staphylococcus aureus* and *Escherichia coli* O157:H7 in chicken egg.

4 Research question

- What type of bacteria is more prevalent on egg shell and egg content?
- Is there a difference of bacterial incidence on egg shell and egg content? If there is what is the reason behind?
- Is there a significant different of bacterial percentage between retail markets and small scale poultry producers?
- What is the contribution of chicken eggs for the emergency of bacteria resistance to antibiotic agents used by human?

5 Material and methods

5.1 Study area

The study was conducted in Addis Ketema sub city, Addis Ababa, Ethiopia which has a total of 297793 people with 144954 male and 152839 females. It covers 9 % of the total population of Addis Ababa. The total area of the sub city is estimated to be 863.85 hector, the total land area of the sub city is 1.66 % from the total area of Addis Ababa and structured in 10 weredas, 28 sub weredas, 84 sefers and 303 blocks. It has 650 estimated numbers of retail markets and 6 small scale poultry producers. This sub city was purposively selected because of their high commercial poultry households and retail markets that make them the hub of poultry industry in Addis Ababa.

5.2 Study design and period

A Cross-sectional study was conducted to assess the occurrence and antimicrobial resistance pattern of *Salmonella spp*., *S. aureus* and *E. coli* O157:H7 from chicken egg in retail market & small scale poultry production in Addis Ketema sub city, Addis Ababa, Ethiopia starting from September to February 2011.

5.3 Samples

5.3.1 Sample source

All chicken eggs during sample collection period between September and January 2011 for 6 months in Addis Ketema retail markets and small scale poultry production system.

5.3.2 Study samples

Selected chicken egg without cracking subjected to bacteriological analysis and antibiotic susceptibility tests.

5.4 Inclusion and exclusion criteria

Visual examination of the eggs

Each egg was thoroughly evaluated visually and placed into one of the following categories: feces and/or blood, dust and/or feathers and total dirty eggs and also the occurrence of cracks and micro-cracks in the eggshell were noted. The visual examination of the eggshell was performed using a candling light.

5.4.1 Inclusion criteria

- Chicken eggs without cracking or micro-cracks
- Eggs which are stored at room temperature

5.4.2 Exclusion criteria

- Eggs looks like clear which are may be washed with special detergent and sanitizer

5.5 Study variables

5.5.1 Dependent variable

- Magnitude of isolated bacteria
- Antibiotic susceptibility and resistance pattern of isolated bacteria

5.5.2 Independent variable

- The color of eggshell (white and brown)
- Site of collection area (retail market and small scale poultry production center)
- Parts of eggs where sample is collected (shell and content)

5.6 Sample size calculation and sampling method

5.6.1 Sample size calculation

The sample size is calculated using 95% confidence interval and 0.05 absolute precision by assuming expected prevalence of 50%. $n= (1.96 /d)^2 \times p (1-p)$. In which:-

n= number of sample size

d = absolute precision

p = expected prevalence /expected proportion

So $n = (1.96/0.05)^2 \times 0.5(1-0.5)$

n = 384

5.6.2 Sampling methods

A multi-stage sampling technique was used. First the sub city was divided in to woredas then counts the number of retail markets in each woreda then based on appropriate allocation determine the number of retail markets used for sample collection in each woreda. After that randomly collect egg samples from the selected retail markets in Addis Ketema sub city, Addis Ababa and 36 pooled egg samples were collected from 6 small scale poultry producers found in Addis Ketema sub city, Addis Ababa, Ethiopia.

The number of retail markets selected for sample collection in each woreda will be determined using the formula:

$n_j = n / N \times N_j$,j=1, 2, 3,……k where, k is the number of woreda and

- n_j is the number of retail markets in the j^{th} woreda used for sample collection
- N_j is total retail markets of the j^{th} woreda
- $n = n_1 + n_2 + ... + n_k$ is the total retail markets used for sample collection
- $N = N1 + N2 + ... + N_k$ is the total retail markets of the sub city

Samples were collected from Addis Ketema sub city. The sub city comprises 10 weredas all of which were selected for sample collection. The number of retail markets selected for sample collection in each woreda were ($woreda_1 = 3$, $woreda_2 = 4$, $woreda_3 = 5$, $woreda_4 = 6$, $woreda_5 = 4$, $woreda_6 = 3$, $woreda_7 = 5$, $woreda_8 = 2$, $woreda_9 = 5$, $woreda_{10} = 3$).

5.6.3 Sampling procedure

Sampling of eggs from retail markets and small scale poultry producers was carried out from September to February 2011. One hundred eight (180) table eggs which comprise 36 (5 eggs per composite) composite samples were collected from 6 small scale poultry producer and two hundred (200) raw eggs (40 composite samples) displayed for sale were purchased from 40 retail markets.

5.7 Measurement and data collection

5.7.1 Laboratory analysis

5.7.1.1 Processing of egg samples

The first stage of microbiological analysis of food consists in taking and preparing a sample for analyses. Incorrect sampling can lead to obtaining false negative or false positive results. When talking about taking samples, the term “representative sample” is often used. The sample should reflect the image of the product from which it originates as precisely as possible.

A total of 380 egg samples comprising 76 composite samples (5 eggs per composite sample) were collected randomly from different retail markets and small scale poultry production system in Addis Ketema sub city, Addis Ababa using sterile plastic bag prepared from biohazard plastics and transported to food microbiology laboratory and processed immidetly after collection. The total table eggs were divided in to white and brown eggs based on the color of the egg shell.

For egg-shell sampling, sterile cotton swabs dipped in sterile buffered peptone water (BPW) was used to swab the entire surface of egg shell. The swabs were directly inoculated into 10 ml BPW in screw-capped bottles and the contents of the buffered peptone water (BPW) were mixed thoroughly using vortex to obtain the stock solution (dilution 10^1).

For egg content sampling, outer surface of the eggs were disinfected by soaking in 0.1 % sodium hypochlorite for 10 minutes and then wiping with surgical gauze soaked in 70 % ethanol and opened around the air sac area by hand (with sanitized plastic gloves) using a sterile forceps. This procedure is used to avoid contamination of the egg contents from the germs colonizing the egg-shell. After this operation, the albumin and yolk content of all five eggs were pooled and homogenized to make one sample of egg content. About 25 g of homogenized samples of egg content samples were aseptically transferred with a sterile pipette to 225 ml of buffer peptone water. The mixture then homogenized using a laboratory shaker for 5 minutes to obtain a 1/10 stock dilution.

The stock solution of both egg shell and content were used to prepare working solutions (10^2, 10^3,) in aseptic conditions after decimal dilutions. To prepare working solution, we transferred 1 ml from the first dilution in to a tube containing 9 ml of the diluent using sterile pipette. Then repeat using a third tube until the desired dilution is obtained.

5.7.2 Microbiological analysis

5.7.2.1 Culture method

Isolation and identification of bacterial pathogens were carried out based on their growth pattern and colony characteristics on selective and differential media. In addition, IMViC, Catalase, coagulase, citrate, Urease and motility tests were carried out according to the standard test procedures to confirm the findings.

The Medias used during microbial analysis were Sorbitol MacConkey agar (SMac), MacConkey agar (Oxoid, CM 0007), XLD (xylose lysine deoxycholate agar), Trypticas soya agar (TSA), Nutrient broth, Rappaport-Vassiliadis broth (RV), and Mannitol salt agar. All Medias were prepared following the manufacturer's instruction and sterilized by autoclaving at 121^0C for 15 minutes. Each sample was serially diluted with sterile buffer peptone water and presumptive, confirmatory and complementary tests were done for identifying bacteria. Four separate steps were followed to detect *Salmonella* most efficiently because *Salmonella* often occur in low numbers, sometimes sub lethally injured and often in the presence of much greater numbers of other bacteria.

Pre-enrichment steps: in order to proliferate and regenerate damaged cells 25 g of sample in 225 ml of buffered peptone water was pre-enriched in a non-selective medium (buffered peptone water) at 37°C for about 18 -24 hours.

Enrichment steps: About 0.1 ml pre-enriched sample is transferred to 10 ml selective enrichment broth (Rappaport-Vassiliadis broth) and incubated at 42°C for about 24 hours.

Selective media: - An aliquot from the selective enrichment broth is inoculated to selective agar Xylose lysine deoxycholate agar (XLD) which was incubated at 37°C for 24 hours.

Confirmatory tests: - Presumptive *Salmonellae* were sub cultured on a suitable Trypticase soya agar and then biochemically verified.

The standard weight of analytical portions of food samples examined for the presence of sanitary and enteropathogenic *E. coli* is 25 g. To isolate Enterohemorrhagic *E. coli O157:H7* first the pre enriched samples were inoculated in to differential MacConkey agar then incubated at 37^0c for 24hrs. Suspected colonies of *E.coli* on MacConkey agar were further confirmed with the help of biochemical tests following bacteriological analytical manual (FDA 2001). The *E. coli* colonies

were sub-cultured on Sorbitol MacConkey agar (oxoid) for *E. coil* O157:H7 strain confirmation. Sorbitol-negative Enterohemorrhagic *E. coli O157:H7* colonies were pale compared to bright pink sorbitol-positive colonies produced by other *E. coli* types.

Gram positive bacteria particularly *S. aureus* were isolated on Mannitol salt agar at 35-37^{0}c for 24hr incubation. Standard microbiological identification of *S. aureus* is based on its ability to clump in plasma via the activity of clumping factor (bound coagulase) (CF) and coagulase (free coagulase). The simultaneous production of CF and coagulase is a characteristic feature of most *S. aureus* isolates, and plays an important role in species classification and microbiological diagnosis.

5.7.2.2 Biochemical test

Biochemical test was used to identify bacteria to the species level based on their biochemical reaction. All suspected *Salmonella spp*. and *E. coli* isolates were subjected to the following biochemical tests for confirmation: Kligler Iron (KIA) test, Indole test, Citrate utilization test, Methyl red test, Vogues Proskauer (VP) test, Lysine iron test, motility test and urease test. All presumptive *S. aureus* colonies (golden yellow) were confirmed using catalase and coagulase tests.

Table 1 Biochemical characteristics of *Salmonella spp.* and *E. coli*

Biochemical tests	Isolated bacteria test results	
	Salmonella spp.	*E. coli*
Kligler Iron (KIA) test	K/A	A/A
Indole test	-	+
Citrate utilization test	+	-
Methyl red test	-	+
Vogues Proskauer	-	-
Lysine iron test	K/K	K/K
motility test	+	+
urease test	-	-

* (+) = positive test, (-) = negative test, K = alkaline, A = acid, K/A= (slant / butte)

5.7.2.3 Antimicrobial Susceptibility Test

Antibiotic sensitivity pattern of the isolates was performed according to agar disc diffusion method on Mueller–Hinton Agar using National Committee for Clinical Laboratory Standard (NCCLS 2018) guidelines. Prepare the medium as instructed by the manufactures and the pH of the medium should be between 7.2 – 7.4. Isolated bacterial colonies were mixed with normal saline and turbidity was matched with 0.5 McFarland turbidity standards. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. Spread the test organism over the Muller-Hinton agar plate using a sterile swab by rotating three times at 60^{o} angle for even distribution of bacteria and then place the antimicrobial disc on the inoculated plate using a sterile needle and incubate at 37°C for 24 hours. The zone of inhibition is measured by millimeter of ruler & values are matched (compared) with the predetermined standard values & reported as susceptible, intermediate and resistant using clinical and laboratory standard institute 2018 guidelines. Commonly prescribed antibiotic discs used for gram negative bacteria were ampicillin (10µg), ceftriaxone (30µg), gentamicin (10µg), tobramycin (10µg), ciprofloxacin (5µg), ceftazidim (30 µg), naldic acid (30µg), chloramphenicol (30µg), tetracycline (30µg), piperacillin (30µg) and cefoxitin (30µg). Erythromycin (15µg), penicillin (10units), cefoxitin (30µg), clindamycin (2µg), gentamicin (10µg), tobramycin (10µg), ciprofloxacin (5µg) and chloramphenicol (30µg) antibiotic disks were used for gram positive bacteria (*S. aureus*) antimicrobial susceptibility test. The criteria used to select the antimicrobial agents were based on the availability and frequency of prescription for the management of bacterial infections in Ethiopia.

5.8 Data Quality Assurance

5.8.1 Pre analytical

All culture media were checked visually before use for contamination, significant physical imperfections (for example, uneven distributions of media, variable amounts of medium in petri dishes/tubes/bottles, color, gross deformation of the surface on the media) and expiry date. Quality Control for commercially prepared microbiological culture media was applied using American Type Culture Collection (ATCC) control strains before use. We did not use plates if they show evidence of microbial contamination, discoloration, drying, cracking or other signs of

deterioration. Plates that were beyond their expiry date were discarded. Sterile containers were used to collect samples.

5.8.2 Analytical

Standard Operating Procedures (SOP) was strictly followed. Quality control was performed on all materials and equipment which were critical to the detection, isolation and identification of microorganisms. This includes Medias, stains, biochemical test reagents and equipment such incubators and autoclaves. Each new lot was quality controlled before use by testing the *E. coli* ATCC standard strains. The quality of medium and the potency of the antibiotic were checked by using control strains (*E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923). Samples were prepared aseptically to ensure that no bacterial contamination is introduced by contact with hands or contact with unsterile surfaces or items. For example Caps and lids from containers were be placed on the workbench but retained in the hand while the sample is being processed. Caps and lids were replaced as soon as possible. When pouring liquid media ensure that the mouth of the bottle does not come into contact with samples or non-sterile items. If this occurs, discard the media. Dehydrated culture media were stored in the dark and dry place. The optimum storage temperature was 15-25 °C (59-77 °F). Dehydrated culture media were hygroscopic so that the containers were always being tightly closed after use to prevent entry of moisture.

5.8.3 Post analytical

Follow the SOP for reporting results with the reference ranges, units and to the correct decimal place and reporting in a timely manner. Records were retained so that previous post-analytic errors can be tracked and potentially prevented in the future with training and use of the SOP. The isolated bacteria were preserving using 25 % glycerol and stored at -20^0c. The raw data were entered and managed in Microsoft Excel work sheet.

5.9 Data analysis and interpretation

Data was analyses using the SPSS statistic software version 23. An initial descriptive statistics using (tables and percentages) was utilized to summarize data and kruskal Wallis testing was performed to assess the association of each variable independently. The prevalence was calculated for all data by dividing positive samples by total number of examined samples and multiplied by hundred. A statically significant association between variables is considered to

exist if the computed p-value is less than 0.05. Finally, the study findings were described in text, tables, charts and graphs.

5.10 Ethical considerations

The study was approved by "Department Research and Ethical Review Committee (DRERC)" of the Department of Medical Laboratory Science, College of Health Sciences, Addis Ababa University. Permission letter was also obtained from the study site. The purpose and procedures of the study was explained to the study site within the study period.

5.11 Dissemination of the result

After conducting the research, the result of the study was submitted to the Department of Medical Laboratory Sciences, College of Health Sciences and Addis Ababa University, Ethiopian Public Health Institute and other concerned bodies.

6 Operational definitions

Candling:- is the process used to determine the quality of egg using light help.

Small scale poultry production system: - is 50 to 500 flock size exotic breeds kept for operating on a more commercial basis

Retail market: - is the sale of goods and services from businesses to an end use (called a customer)

Composite egg sample: - is a sample which contains more than one eggs mixed in one container used as a one sample in our study.

Table eggs: are eggs in the form of most familiar to consumers fresh and in the shell.

7 Results

7.1 General description

A total of 380 chicken eggs collected randomly from Addis Ketema Sub city (retail markets and small scale poultry producer center), 40 composite samples from retail market and 36 composite samples from small scale poultry producer center were tested by conventional culture method, biochemical and serological testes to detect the magnitude of *Salmonella spp*., *S. aureus* and *E. coli* O157:H7. Both egg shell and egg content were subjected to culture for bacterial isolation.

7.2 Magnitude of *Salmonella spp*., *Staphylococcus aureus* and *E. coli* O157:H7 in eggs sample

The result of microbiological analysis of 76 composite egg samples revealed that 27 % of the egg samples had bacterial contamination distributed on the shell and content as follows: *Salmonella spp*., 14(9.2 %), *S. aureus* 9(5.9%) and *E. coli* O157:H7 18(11.8 %) (Table 1). Out of the total isolates, 80.5 % were from eggs purchased in the retail markets while 19.5 % were isolated from small scale poultry producer. It was confirmed that the retail market eggs are more contaminated compare to small scale poultry producer.

Table 2 Magnitude of suspected pathogenic bacteria isolated from total egg sample from September to February, 2011

Total no. of composite sample examined	Type of bacteria isolates	No. (%) of positive sample	shell	content
76	*Salmonella spp.*	14 (9.2)	11	3
	S. aureus	9 (5.9)	7	2
	E. coli O157:H7	18 (11.8)	14	4
	total	41(27%)	32	9

The highest percentage of bacterial contamination was noted on egg shell surfaces 32 strains (78 % of all isolates), of which 81.3 % were from the retail markets and 18.7 % from the small-scale poultry producers. Out of the total 32 isolated pathogenic bacterial strains on the surface of eggshell *E. coli* O157:H7 (n=14; 18.4 %) was the most predominant isolate followed by *Salmonella spp*. (n=11, 14.5 %) and *S. aureus* (n=7; 9.2 %).

A total of 9 bacterial pathogens were isolated from egg content as *Salmonella spp*. 3(3.9 %), *S. aureus* 2(2.6 %) and *E. coli* O157:H7 4(5.3 %). Out of the total egg content contamination 77.8 % for retail markets compared to 22.2 % for small scale poultry producer. In our present finding it was observed that the highest percentage of bacterial pathogens present in egg content was *E. coli* O157:H7 5.3 % (n= 5) than other pathogens.

Out of the total 76 composite chicken eggs examined for isolation of *Salmonella spp.*, 14.5 % (n= 11) and 3.9 % (n= 3) of egg shells and egg contents, respectively were positive (Tables 2 & 3). The percentage of *Salmonella Spp*. isolated from retail market egg shells was 20 % (n= 8) while 8.3 % (n= 3) were from small scale poultry producer egg shells. In our study we isolated *Salmonella spp*. from 7.5 % (n= 3) of content of eggs purchased from retail markets whereas *Salmonella spp*. was not isolated from the content of eggs taken from small scale poultry producer.

Table 3 Source-wise percentage of Salmonella spp.in egg shell from September to February, 2011

source of egg	Number of shell examined	No. (%) of shell positive
Retail markets	40	8 (20)
Small scale producer	36	3 (8.3)
Total	76	11 (14.5)

Table 4 Source-wise percentage of *Salmonella spp*. in egg content from September to February, 2011

Source of egg	Number of content examined	No (%) of content positive
Retail market	40	3 (7.5)
Small scale producer	36	0 (0)
Total	76	3 (3.9)

On the basis of cultural and biochemical characteristics, 5.9% (n = 9) chicken egg samples were found to be associated with *S aureus*. Among these 9 isolates of *S. aureus*, 7 isolates were obtained from egg shell surface (9.2 %) and 2 isolates were from the egg contents (2.6 %). From the retail markets alone, 40 composite samples (200 eggs) were collected. Out of which 7 (17.5 %) were positive for *S. aureus* from shell while 1 (2.5 %) were positive for *S. aureus* from content. From the small scale poultry producer, 36 composite samples (180 eggs) were collected. Out of which only 1 (2.8 %) yielded *S. aureus* from the content (Table 4).

Table 5 Isolation rate of *S. aureus* in retail markets and small scale producer based on parts of egg sample from September to February, 2011

Sample source	Number of sample	No (%) of *S. aureus* isolated	
		Shell	Content
Retail market	40	7 (17.5)	1 (2.5)
Small scale producer	36	0 (0)	1 (2.8)
Total	76	7 (9.2)	2 (2.6)

The results regarding the incidence of *E. coli* O157:H7 in different collection area has been summarized in Table 5. Out of 40 composite egg samples examined from retail market the incidence of *E. coli* O157:H7 was found in 35 % composite eggs. Similarly, 36 composite eggs acquired from small scale poultry producer, the percentage *E. coli* O157:H7 was observed as 11.1 %.

The results regarding the incidence of *E. coli* O157:H7 in different components of eggs has been summarized in Table 6. Out of the 76 egg shells examined, the incidence was recorded as 18.4 %. Similarly, among 76 egg contents, the incidence of *E. coli* O157:H7 was noted as 5.3 %. *E. coli* O157:H7 was found on the shell surface of 27.5 % of eggs purchased from retail market and on the shell surface of 8.3 % of eggs tested from the small scale poultry producer. There were four *E. coli* O157:H7 isolated from egg content (22.2 % of all *E. coli* O157:H7 isolates), of which 75 % (n=3) were isolated from eggs purchased from retail market and 25 % (n=1) from eggs in the small scale poultry producer. *E. coli* O157:H7 was recovered at a higher frequency from the surface of the shells than the egg contents.

Table 6 The number and percentage prevalence of *E. coli* O157:H7 in table eggs collected from different sites from September to February, 2011

Collection site	No. of examined sample	No. of positive components	% of positive components
Retail market	40	14	35
Small scale producer	36	4	11.1

Table 7 The number and percentage incidence of *E. coli* O157:H7 in different components of table eggs from September to February, 2011

Egg components	No. of egg component examined	No. of positive components	% of positive components
Egg shell	76	14	18.4
Egg content	76	4	5.3

The pie chart presented in Figure 1 shows the percentage distribution of *Salmonella spp., S. aureus and E. coli* O157:H7 contamination in white and brown color of egg. Out of the total 76 composite sample, 29 % isolated bacteria were found from white and 25 % were isolated from brown egg samples. In this case, *E. coli* O157:H7 had the most incidence 11.4 % followed by *Salmonella spp*. and *S. aureus* in both white and brown egg samples.

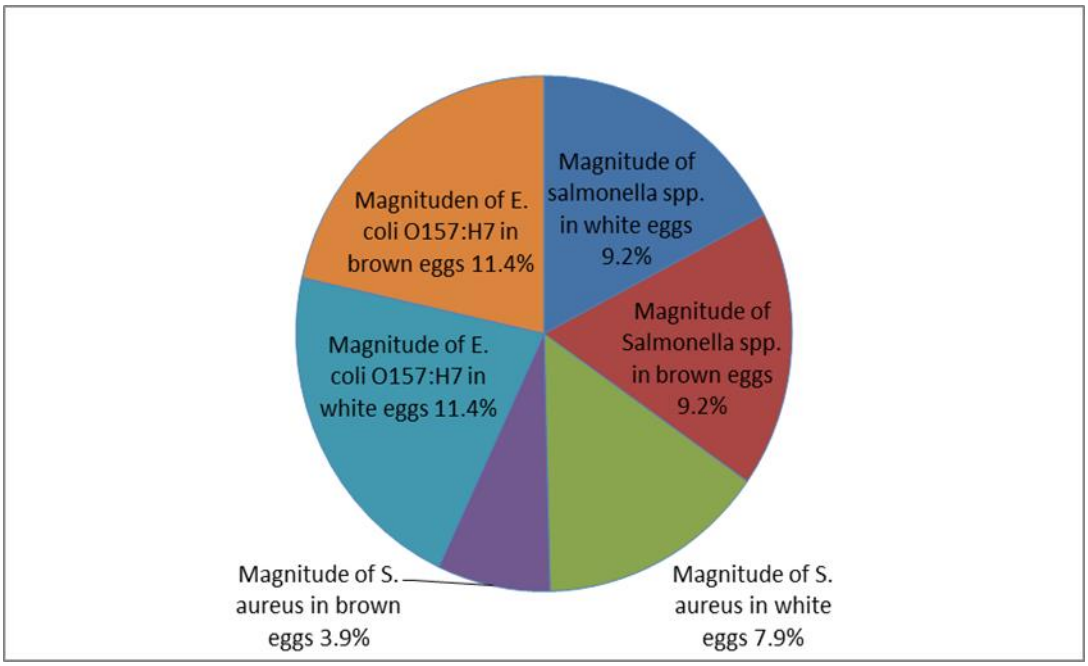

Figure 1 Magnitude of isolated bacterial contaminations of chicken eggs based on their color of shell from September to February, 2011

7.2 Antibiotic resistance patterns of *Salmonella spp., S. aureus* and *E. coli* O157:H7 in chicken eggs

Antimicrobial susceptibility tests were done on total of 41 isolates (14 *Salmonella spp*., 18 *E. coli* O157:H7 and 9 *S. aureus*). The antimicrobial susceptibility test result showed that all the

isolates were resistant to at least one antibiotic in the disc diffusion test. All the isolates were susceptible to chloramphenicol.

Salmonella antimicrobial results are summarized in figure 2. All isolates of *Salmonella* (100 %) were susceptible to three antimicrobial agents, namely, naldic acid, piperacillin and chloramphenicol. *Salmonella spp.* showed the highest resistance patterns against ampicillin (71.4 %), cefoxitin (42.9 %), ciprofloxacin (42.9 %) and ceftazidim (35.7 %). The least resistance rates were recorded against tetracycline, ceftriaxone, gentamicin and tobramycin; 14.3 %, 7.1 %, 7.1 % and 7.1 % respectively.

During present experiments, Eleven (11) different antibiotics were tested to demonstrate the in-vitro susceptibility of *E. coli* O157:H7 isolates recognized from the table eggs and results were given in figure 2. Antimicrobial agents for which many *E. coli* O157:H7 isolates exhibited resistance were ampicillin (72.2 %), cefoxitin (55.6 %) and tetracycline (27.8 %), on the hand all *E. coli* O157:H7 isolates were sensitive to tobramycin, chloramphenicol and ciprofloxacin. *E. coli* O157:H7 were resistant at various proportions to the other tested antibiotics disks such as ceftazidim16.7 %), nalidic acid (16.7 %), ceftriaxone (5.6 %) and gentamicin (5.6 %).

Table 8 Antibiotic resistance profile of *E. coli* O157:H7 and *Salmonella spp*. isolates from egg samples collected from September to February, 2011.

Antimicrobial agents	% resistance of Salmonella spp.	% resistance of E. coli H157:H7
AMP	64.3	72.2
CHL	0	0
CIP	35.7	0
GEN	7.1	5.6
TET	14.3	27.8
CAZ	28.6	16.7
CXT	35.7	55.6
NAL	0	16.7
PIP	0	16.7
TOB	7.1	0
CTR	7.1	5.6

AMP=ampicillin, CHL=chloramphenicol, CIP=ciprofloxacin, GEN=gentamycin, TET=tetracycline, CAZ=ceftazidim, CXT=cefoxitin, NAL=nalidic acid, PIP=piperacillin, TOB=tobramycin, CTR=ceftriaxone

Antimicrobial resistance pattern of *S. aureus* isolated from table egg to different antibiotics were summarized in table 7. All (100 %) the *S. aureus* were resistant to tetracycline whereas, all *S. aureus* strains isolated from chicken eggs were susceptible to gentamicin and chloramphenicol. High resistance was observed against oxacillin (88.9 %), penicillin (77.8 %), clindamycin (44.4 %), erythromycin (44.4 %) and ciprofloxacin (33.3 %).

Table 9 Resistant pattern of *S. aureus* bacteria isolated from egg sample from September to February, 2011.

Antimicrobial agents	Resistant *S. aureus* n (%)
TET	9 (100)
OXN	8 (88.9)
PEP	7 (77.8)
DA	4 (44.4)
E	4 (44.4)
CIP	3 (33.3)
GEN	0 (0)
CHL	0 (0)

PEP=penicillin, DA=clindamycin, GEN=gentamycin, E=erythromycin, CIP=ciprofloxacin, TET=tetracycline, OXN=oxacillin, CHL=chloramphenicol

8 Discussion

Regarding the increasing consumption of egg and its product, it is necessary to investigate egg contamination. Eggs and eggs products had been described as one of the main food vehicles associated with foodborne outbreaks (79). The safety of eggs depends on the number of bacterial cells on the shells and in the content of the egg and on the rate at which they multiply within it (80). The contamination may have resulted from both horizontal and vertical infection, since contamination in eggs is as a result of exposure to environmental conditions (for example, soil, dust and dirty nesting materials), eggs become contaminated with different types of microorganisms (81). The risk of illness resulting from consumption of contaminated eggs depends not only on the number of bacterial cells in eggs, but also on the type of bacteria (82). Among the common contaminant organisms pathogenic to human beings are *Salmonella spp*., *S. aureus* and *E. coli* O157:H7 (47). The isolated bacteria could cause severe health problems like, diarrhea, nausea and abdominal pain since they are pathogenic.

The overall percentage of *Salmonella spp*. in our present study was 9.5 %. These findings are in agreement with the report of Minte A. et al. (2011) (72) in kombolcha, Ethiopia and Okorie O .et al. (2016) (83) in Enugu state, Nigeria they indicated that prevalence of *Salmonella* was 11.5 % and 10.3 % respectively. A survey done in the North West of England and London reports 9.3 % *Salmonella spp*. was isolated from the egg sample (84). However, the prevalence found in our study was comparatively higher than the findings of Ambalang AR. et al. (2017) (52) in Tshwane, South Africa, Jelalu K. et al. (2016) (85) in Haramaya, Ethiopia and Waleed Al. et al. (2018) (86) in Jordanian Markets who observed the prevalence was 6.8 %, 2.7 % and 6 %, respectively.

Our data indicated the frequency of *Salmonella spp.* on the surface of egg shell were 14.5%. This study results are in agreement with the results of Ifeanyichukwu I. et al. (2018) in Ebonyi State, Nigeria (26) 16.3 %, Dawit N. et al. (2015) in Mekelle, Ethiopia (71) 15.38 % and Arufa P. et al. (2017) in Dinajpur district (70) 27.78 %. However, a study done by long D. et.al (2018) has reported that, prevalence of *Salmonella spp.* in egg shells were 3.6 % which was lower than our study (78). The prevalence of *Salmonella spp.* on egg shells was 5.4 % in Trinidad and Tobago (65) and 6.1 % in Kombolcha, Ethiopia (72) were lower than that of the present finding. In contrast to our current study Mahdavi M. et al. (2013) (11) and Sabry H. et al. (2012) (9) were

not detected *Salmonella spp*. on the egg shell surface in Iran. It reflects may be the different sanitary measures adopted during production, handling and storage conditions in different country.

The overall prevalence of 3.9 % for *Salmonella spp.* in table egg contents in the present study was lower than the prevalence of 9.2 % reported in Trinidad and Tobago (65), 11.1% in Bangladesh (70) and 6.8 % in Kombolcha, Ethiopia (72). In contrast to our result, other researchers indicates there was no contamination of *Salmonella spp.*, in the content of egg (42, 74).

This study showed that there is a significant difference in the prevalence existent of *Salmonella* contaminants depending on the sampling location of the egg (from the shell and content of eggs). In our present study, the rate of *Salmonella spp.*, from eggshell (14.5 % v_s 3.9 %) is higher than egg contents and the difference is stastically significant ($p = 0.025$). Similar findings published by Minte A. et al. (2011) in Ethiopia (72) have been reported in studies involving the isolation of *Salmonella spp.*, from the egg shell and content of chicken eggs. On the other hand, Salihu M. et al. (2015) in Sokoto metropolis, Nigeria (77) and Adesiyun A. et al. (2005) in Trinidad (65) reported that isolation rate of *Salmonella spp.* was higher in the contents of the egg samples than the shell surfaces. Considerable variation may be related to antimicrobial defenses of the egg content and eggs shell are exposed to contamination due to bad storage conditions in storehouse, wrong show in market, dirty table, dust, hand touching, and all other surrounding pollution.

The overall percentage of 5.9 % for *S. aureus* in the present study is considerably lower than the percentage of 7.2 % and 10.45 % reported by Ali A. et al. (2015) in Thailand (6) and Pondit et al. (2018) in Mymensingh district (74) respectively. In another study, reported by Jannatul F.et al. (2016) from different markets of Dhaka city 66.67 % (69) of the isolated bacteria from egg sample were covered by *S. aureus* which was higher than what we found in the present study. Eman F. et al. (2015) (22) Found a high degree of contamination of table eggs with coagulase positive S*taphylococcus* bacteria from different markets in Cairo, Egypt. Thus, variation in the prevalence of *S. aureus* in egg might be due to inappropriate storage condition at market level.

Our results reveal that *S. aureus* from egg shell recorded higher contamination than egg content, even though it was not statistically significant ($p=0.087$). In Poland Marek A. et al. (2013) (88) Also indicate greatest number of *S. aureus* strains (55.5 %) were isolated from the shells, while

27.8% of isolates were obtained from the yolks. Although the bacteria were widely distributed on both eggshell and content, there was a higher percentage contamination on the egg shell, these differences may be because of the shell is the most exposed part of the egg to the environment, potentially comes into contact with a wider variety of bacteria than the internal content. In developing countries like Ethiopia, an increased percentage of bacterial contamination have been found on egg surface may be due to inappropriate refrigeration and even no refrigeration during the market storing.

In our study the frequency of *E. coli* O157:H7 on eggshell surface (18.4 %) and egg content (5.3%) was statistically significant (p=0.012). In Trinidad Adesiyu A. et al. (2005) had reported that the prevalence of *E. coli* O157:H7 on egg shell was significantly higher than that found in egg contents (65). Atoyebi A. et al. (2018) in Ibadan (89) report the incidence of *E. coli* O157:H7 was 7 (9.8 %) which is comparatively lower than our result it may be because of serological test was done for identification of *E. coli* O157:H7.

The presence of *E. coli* O157:H7 on chicken egg suggests that chickens may be natural carriers of the microorganism, or contamination may be coming from other sources such as exposure to environmental conditions for example, soil, faces and dirty nesting materials. Several factors were implicated in egg contamination due to the principle way of contamination occurs in short time after laying as the egg shells become within contact environment as soil, dust and dirty nesting material (90). Most of bacterial contamination of eggs contents results from bacterial contamination of egg shells that invade egg contents under improper conditions, beside the horizontal way egg may contaminated vertically or through ovary and oviduct (91).

E. coli O157:H7 in chicken egg determined in the present study, suggests that chicken egg may be a source of human infections due to this organism. *E. coli* O157:H7 is regarded to maintain its natural long life cycle in the environment by its resistance to cooling, freezing and to acidic conditions (92). Therefore, *E. coli* O157:H7 draws attention, due to its potential to produce severe, life-threatening illness in humans, and its low minimal infective dose.

In this study, it was observed that eggs from retail markets (13.8%) recorded significantly higher contamination ($p<0.042$) with *Salmonella spp*. than eggs obtained from small scale poultry producer (4.2%). In Haramaya, Ethiopia Kemal et al. (2016) report that the level of *Salmonella spp.* in the local market was significantly higher than the level of *Salmonella spp.* in the poultry

farm (p = 0.007) (85). This might be because of unhygienic conditions in the retail markets where these eggs are openly displayed for sale, the improper/unhygienic handling of eggs by farm workers, retailers and buyers, the prolonged poor storage conditions of the eggs during sale.

However, our result disagrees with the result of Adesiyun et al. (2005) in Trinidad who reported significantly higher contamination of the eggs from farms with *Salmonella spp*. than retail markets (93). The reason for this could be due to the additional washings of eggs carried out at the sale outlets in the study area to make the eggs appear clean and acceptable to the consumers which are not done in Ethiopia.

The total frequency of *S. aureus* varied among the sampling sites. The prevalence of *S. aureus* in the retail markets (10 %) was significantly higher than the prevalence of *S.aureus* in the small scale poultry producers (1.4 %) (p = 0.025). High percentage of contamination of eggs collected from retail markets may be attributed to the fact that the eggs were stored under room temperature for many days until purchase.

The frequency of contamination of *E. coli* O157:H7 was significantly higher (P = 0.023) in egg samples collected from retail markets (17.5 %) than from small scale producer (5.6 %).

Antimicrobial resistance is an increasingly global problem and emerging antimicrobial resistance has become a public health issue worldwide (94). A variety of foods and environmental sources harbor bacteria that are resistant to one or more antimicrobial drugs used in human or veterinary medicine and in food-animal production (40). The consumption of infected eggs (raw or uncooked) may cause different food-borne diseases which can be treated only by the use of chemotherapeutic agents. Increasing use of antibiotics in the poultry industry is a concern since it increases bacterial exposure to antibiotics in relatively low doses, increasing the risk of development of antibiotic resistance by the bacteria (2). Unluckily, the development of bacterial resistance to antibiotics quickly reduced ability of a particular antibiotic, so it is necessary to test pathogens against various antibiotics to determine susceptibility or resistance to that drug.

All of the 9 isolates of *S. aureus* from chicken egg sample were subjected to antibiotic sensitivity test against commonly used antibiotics. Antimicrobial susceptibility analysis result showed *S. aureus* was resistant to several antibiotics. Here, *S.aureus* isolates from chicken eggs showed varying degrees of resistance to penicillin (77.8 %), erythromycin (44.4 %) and ciprofloxacin (33.3 %). Similar results reported by Pondit A et al. (2016) (95) indicated that 82.6 %, 39.13 %

and 26.8 % isolates of *S. aureus* were resistant to penicillin, erythromycin and ciprofloxacin respectively. In our study resistance rate of *S. aureus* against erythromycin (44.4 %) was relatively higher to the result of Nazer A. et al. (2016) in Shiraz, Iran (63) who reported 37.5 % whereas, Marek A. et al. (2013) (88) in Poland report highest number of *S. aureus* strains were resistant to erythromycin (66.66%). The resistant pattern of *S. aureus* against oxacilin (88.9%) and tetracycline (100%) in our study was comparatively higher than Jannatul F. et al. (2016) in Dakar (69) and Pondit A. et al (2016) in Bangladesh (95). In the present study, all *S. aureus* strains were resistance to tetracycline. Pyzik E. et al. (2013) (88) reported that *S. aureus* isolates were resistance to tetracycline (66.66 %), which was lower than our study.

In the present study, *E. coli* O157:H7 isolates were observed to be the most resistant to ampicillin, cefoxitin, tetracycline and nalidic acid (72.2 %, 55.6 %, 27.8 % and 16.7 %) respectively. None of the isolates were resistance for chloramphenicol, piperacillin and tobramycin. Comparative study by Mahfuzul et al. (2018) in Dhaka city reported that, resistance of *E. coli* O157:H7 for tetracycline, chloramphenicol and nalidic acid (100 %, 38.89 % and 22.22 %) were higher than our study but the resistance pattern of *E. coli* against Ceftriaxone in the present study have close agreement with the finding of Mahfuzul et al.(2018) (67). In our antibiotic susceptibility test conducted on the *E. coli* O157:H7 isolated from the egg samples reveal that all the isolates were susceptible to ciprofloxacin which is similar with the results of Khan A et al.(2016) (96). The use of antibiotics as prophylaxes, growth promoters or inaccurate dosages given to sick flocks by unqualified personnel, which might be the possibly reason for the occurrence of resistance profile of isolated bacteria.

9 Limitations of the study

- ✓ The egg surface and egg content may possess large number of microorganisms but the study was restricted to three most common medically important pathogenic bacteria (i.e. *Salmonella spp., E. coli* O157:H7 and *S. aureus*) because of time constraint and lack of reagents necessary to do all.
- ✓ The study was restricted to Addis Ketema sub city only.
- ✓ Serological test was not performed for confirmation of the isolated *E. coli* O157:H7 because of lack of *E. coli* O157:H7 antisera.

10 Conclusion

Based on the results of the study, it can be concluded that raw table eggs marketed for human consumption in Addis Ketema, Addis Ababa Ethiopia is contaminated with foodborne pathogens *Salmonella spp*., *S. aureus* and *E. coli* O157:H7. The isolated pathogens were resistance to commonly used antimicrobial agents in veterinary and human practices. Hence may pose a health hazard to human beings if consumed improperly cooked or raw eggs. This could have a significant public health consequence if these microorganisms are transmitted to humans through food chain.

11 Recommendations

- ✓ No single universal mitigation option capable of eliminating contamination of egg is currently feasible. It is important to human welfare that contamination is controlled throughout the whole egg production chain from the farm to the table. Controlling foodborne pathogens throughout the food production chain enables producers to reassure consumers that the foods they are purchasing are safe and wholesome.
- ✓ To minimize the incidence and spread of microorganisms of public health significance; the government should set National Farm Biosecurity Manual to prevent or control the introduction and spread of infectious agents to a flock. Hence, the prevention or reduction of these pathogenic agents in the intestines of hens may greatly reduce contamination of table eggs which has public health importance.
- ✓ The presence of pathogenic bacterial contamination in local chicken eggs is of public health concern, as these are the most widely available and used egg types. Therefore, the public and health officials should be made aware of risks associated with consumption of

raw chicken eggs contain pathogenic bacteria and marketers should aware to ensure good hygienic standard at various retail outlets to minimize bacterial contamination of egg.

- ✓ The government should set policies ‘any product in which raw eggs are used must be provided with a conspicuous label stating that it may contain pathogenic bacteria’. Due to these concerns, public perception of a “good egg” may be shifted from shell cleanliness and physical properties to that of microbial integrity.
- ✓ Minister of health should encourage the education of farmers and training of food handlers and consumers in food safety as the pivotal point of preventing salmonellosis.
- ✓ In some cultures, people eat raw or uncooked eggs. This practice could predispose the people to foodborne infections such as salmonellosis. Therefore, it is advisable to cook any egg and egg product before consumption, rather than consuming as a raw.
- ✓ Poultry industries should perform periodic antibiotic susceptibility tests and carful use of antibiotics in poultry industry which are essential for the control of further emergence of multidrug resistance.
- ✓ Majority of the isolates have developed drug resistance endangering poultry production and public health as these drugs are used widely for treatment and prophylaxis in animals and humans. Therefore, future research should be focused on molecular characterization for serotyping genes responsible for pathogenicity and drug resistance.

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13 Annex I

10.1 Lab SOPs

10.1.1 Sample collection

If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be meaningless. Because interpretation about a large consignment of food is based on a relatively small sample of the lot, established sampling procedures must be applied uniformly. A representative sample is essential when the microorganisms are sparsely distributed within the food. Moreover, the condition of the sample received for analysis is of primary importance

All samples were aseptically collected using sterile plastic bag (for dry, unfrozen materials only) and take to the laboratory for microbial analysis with a minimum of delay for immediate examination. The right sample received at the right time.

Arrival at the laboratory. As soon as the sample arrives at the laboratory, the analyst should note its general physical condition.

Condition of sampling container. Check sampling containers for gross physical defects. Carefully inspect plastic bags used for sampling, be certain that twist wires did not puncture surrounding bags. Any cross-contamination would invalidate the sample.

Labeling and records. Be certain that each sample is labeled appropriately and is accompanied by a completed copy of all records

Preparation of sample homogenate. Make a 1: 10 dilution of the well mixed sample, by aseptically transferring 25gm of sample to 225ml of buffered peptone water. Pipette 1ml of sample homogenate in to a tube containing 9ml of the diluent. Repeat using a third, fourth or more tubes until the desired dilution is obtained.

10.1.2 Preparation of culture media

Re-hydrate powder according to manufacturer's instructions. Before sterilization, ensure ingredients are completely dissolved, using heat if necessary. Avoid wastage by preparing only sufficient for either immediate use **(**allowing extra for mistakes**)** or use in the near future.

Major steps in preparation of culture media:

- ✓ Weighting & dissolving of culture media

- ✓ Sterilization of media
- ✓ Addition of heat sensitive ingredients (optional)
- ✓ Dispensing of media
- ✓ Labeling & storage of media
- ✓ Quality assurance: testing for sterility, pH & performance of media

Weighing & dissolving of ingredients

- ✓ Exactly follow manufacturer's instructions written on the container
- ✓ Weigh accurately with analytical balance in a cool, clean & dry environment
- ✓ Use distilled water
- ✓ Add powder to water & mix by rotating or stirring the flask. Do not shake
- ✓ Stir while heating if required to dissolve the medium – avoid boiling & foaming

Overheating may alter the nutrients, pH & gelling property of the medium.

Dehydrated culture media are in principle not safe. They contain hazardous/ toxic substances like bile salts, azide, selenite, dyes, etc. and powder. It is advised to take the precautions preventing the exposure to powdered dehydrated culture media. The inhalation of dust from powder spread during weighing may be hazardous and must be prevented.

A Good Laboratory Practice (GLP) preparation of culture media requires that during heating evaporation of the reconstituted medium is prevented. Evaporation does not only change the concentration of the ingredients in the reconstituted medium but vapor coming from the media may contain hazardous/toxic substances.

Sterilization

Sterilization is a procedure designed to entirely eliminate viable microorganisms from a material or medium. Sterilization can be accomplished by:

Autoclaving

Autoclaving is a process used to destroy microorganisms and decontaminate bio hazardous waste and microbiological equipment used at Biosafety Level 1, 2, 3 and 4.

Autoclaves use high pressure and high temperature steam for sterilization. The potential safety risks for the operators include:

- ✓ Heat burns from hot materials and autoclave chamber walls and door.
- ✓ Steam burns from residual steam coming out from autoclave and materials on completion of cycle.

- ✓ Hot fluid scalds from boiling liquids and spillage in autoclave.
- ✓ Hand and arm injuries when closing the door.
- ✓ Body injury if there is an explosion.

Addition of heat sensitive ingredients

- ✓ Refrigerated heat sensitive ingredients should be first warmed at room temperature before added to a molten agar medium.
- ✓ Using an aseptic technique, the ingredient should be added when the medium has cooled to 45^0c & should be distributed immediately before the medium starts to solidify.

Dispensing of culture media

- ✓ Media should be dispensed in a clean, if possible sterilized room
- ✓ Most fluid media, unlike solid media, are first dispensed into screw capped bottles or tubes, & then sterilized by autoclaving

Procedure:

- ✓ Lay out the sterile petridish on a level surface - lab bench
- ✓ Mix the medium gently by rotating the flask or bottle. Avoid air bubbles
- ✓ Flame sterilizes the neck of the flask & pour 12 – 15ml of medium into each petridish
- ✓ When the medium has gelled & cooled, stack the plates & tubes & seal them in plastic bags, to prevent loss of moisture & reduce the risk of contamination & Store at 2 – 80c

Note: if air bubbles enter while pouring, rapidly flame the surface of the medium before gelling occurs.

- ✓ Rotate the dish on the bench to ensure an even layer of agar
- ✓ Sterilized agar media in a screw cap bottle can be set in a slope or slant position to increase the surface area for aerobic inoculation

Labeling & storage

All culture media should be clearly labeled with:

- ✓ Its name e.g. Mac, SS, BA
- ✓ Date of preparation and expiration

Media should be stored at 2 – 8^0c, in sealed plastic bags to avoid drying, protected from direct sunlight

Media should also be warmed to room temperature before use.

Quality control of media

Culture media should be checked for their pH, sterility & performance

PH testing

- ✓ The pH of most culture media is near neutral, except peptone water w/c is alkaline.
- ✓ The pH is tested by using a narrow range pH papers or a pH meter.
- ✓ A fluid medium can be tested by dipping a pH paper into a sample of the medium at room temperature & color is compared with the pH color chart.
- ✓ An agar medium can be tested by laying a pH paper on its surface
- ✓ The pH of a dehydrated medium may need to be adjusted when it is prepared & ready for use.
- ✓ The pH of media can be adjusted using 0.1M (N) NaOH and 0.1M (0.1N) HCl.

Sterility testing

Incubate one tube or plate from each autoclaved or filter-sterilized batch of medium overnight at 35˚–37˚C and examine it for contaminants. Culture media may be contaminated with environmental contaminates if aseptic technique is not employed. Contamination of media can be shown by turbidity in a fluid medium & growth and formation of colony on or in a solid agar medium.

Performance testing

Use at least one strain to test for ability of selective media to support growth of the target pathogen (e.g., for MacConkey agar, a *Shigella* strains such as S. flexneri). Documentation should be made regarding whether this strain produces the appropriate biochemical reactions / color on the test medium.

A set of control organisms (stock strains /culture) are required & viability should be maintained with regular sub culturing.

Ability to produce appropriate biochemical reactions

For selective media: Use at least one organism that will grow on the medium and at least one organism that will not grow on the selective medium to test for the medium's ability to differentiate target organisms from competitors. If the medium is both selective and differential, it may be useful to include two organisms that will grow on the medium and produce different reactions (e.g., for MacConkey agar: a lactose nonfermenting organism such as S. flexneri; a lactose-fermenting organism such as E. coli; and, S. aureus, which should not grow).

• **For biochemical media:** Use at least one organism that will produce a positive reaction and at least one organism that will produce a negative reaction (e.g., for urea medium, a urease-positive organism such as Proteus and a urease negative organism such as E. coli).

Sources of quality control strains

Suitable QC strains may be obtained in the following ways.

- ✓ A laboratory may use strains isolated from clinical specimens or quality assurance specimens, provided the strains have been well characterized by all available methods (e.g., biochemical, morphologic, serologic, and molecular).
- ✓ Many laboratories purchase QC strains from official culture collections (e.g., the American Type Culture Collection [ATCC] and the National Collection of Type Cultures [NCTC])

Quality control of reagents

As with all other products used in testing, reagents (whether purchased or prepared in the laboratory) should be clearly marked to indicate the date on which they were first opened and the expiration date, if appropriate. Each reagent should be tested to make sure the expected reactions are obtained.

If the reagent is a rare, expensive, or difficult-to-obtain product (e.g., diagnostic antiserum) it does not necessarily have to be discarded on the expiration date.

If satisfactory sensitivity and specificity can still be verified by normal QC procedures, the laboratory may indicate on the vial label the date of verification of quality of the reagent. All reagents should be tested for quality at intervals established by each laboratory to ensure that no deterioration has occurred; if the quality of the reagent is being verified after the expiration date, testing should be performed more frequently.

Slide agglutination method for quality control of antisera

For QC of antiserum, two or more control strains (one positive and one negative) should be used to test the agglutination characteristics of the antiserum. The results of all reactions should be recorded. Following is an example of a typical QC procedure.

- ✓ Place a drop (about 0.05 ml, though as little as 10 µl can be used) of each antiserum on a slide or plate. Also, place a drop of 0.85% saline on each slide or plate to test each antigen for roughness or autoagglutination.
- ✓ Prepare a densely turbid suspension (2 or 3 McFarland turbidity standard) of each control isolate in 0.85% saline with growth aseptically harvested from an 18- to 24-hour culture from nonselective agar (e.g., HIA or tryptone soy agar [TSA]).
- ✓ Add one drop of the antigen suspension to the antiserum and the saline. Mix thoroughly with an applicator stick, glass rod, or inoculating loop. Rock the slide back and forth for 1 minute.
- ✓ Read the agglutination reaction over a light box or an indirect light source with a dark background. The saline control must be negative for agglutination for the test to be valid.

10.1.3 Inoculation and identification of culture media

There are two major types of inoculation techniques are used in food microbiology laboratory to inoculate the samples in different culture media**.**

Streak plate method:

Spreading the colonies throughout four quadrants in such a way that the four quadrants will have the least amount of bacteria with in it.

Streaking procedure

1. Hold the inoculating loop handle in one hands as you would a pencil; hold the agar plate in the opposite hand
2. Open the lid of the agar plate enough to insert the spread the inoculum over the surface of one quadrant of the agar plate ;do not streak over previously streaking line
3. Flame and cool the inoculating loop
4. Turn the plate so section two is in position ;streak the second quadrate of the plate by touching the loop in to the first quadrate and streak all the way across the second quadrate
5. Flame and cool the inoculating loop
6. Streak the third quadrate by touching the loop in to the second quadrate and streak in to the third quadrate
7. Flame and cool the inoculating loop

8. Streak the fourth quadrate in a manner to produce isolation colony ; touch the loop to the third quadrate and spread the organism in to the fourth quadrate
9. Flame and cool the inoculating loop

Pour plate method:

is usually the method of choice for counting the number of colony forming bacteria present in the liquid specimens .in this method fixed amount of inoculum (generally 1ml) from a broth /sample is placed in the center of sterile petri dish using a sterile pipette . Molton cooled agar (approx. 20ml) is then poured in to the petri dish containing the inoculum and mix well .after the solidification of the agar, the plate is inverted and incubated at 370c for 24 -48hrs

Microorganisms will grow both on the surface and within the medium .colonies that grow within the medium generally are small in size and may be confluent; the few that grow on the agar surface are of the same size and appearance as those on the streak plate .each colony is carefully counted (using magnifying colony counter if needed).each colony represent a "colony forming unit" (UFU)

The pour plate method of counting bacteria is more precise than the streak plate method, but on the average, it will give a lower count as heat sensitive microorganisms may die when they come contact with hot, molten agar medium. The base of the plate must be covered, agar must not touch the lid of the plate and the surface must be smooth with no bubbles.

Streaking and pouring the sample aseptically into the prepared media and see the growth characteristics of microorganisms for identification.

14 Annex II

14.1 Common culture media

XLD agar contains lactose, saccharose, L-lysine, sodium thiosulphate, sodium deoxycholate, ferric ammonium citrate (III) and phenol red. Differential agents of the agar include: lactose, saccharose, xylose, lysine and sodium thiosulphate, from which hydrogen sulfide is released, forming in reaction with iron salts (III) black residue of iron sulfide in the center of the colony. The pH indicator is phenol red. The agar makes it possible to determine the sugar fermentation ability. Incubation is carried out at 37ºC for 24±3 hours. Typical colonies can be colourless, very light, slightly shiny and transparent (colour of the medium) with a dark tinted center, surrounded by a light red area and yellow edge, or of pink to red colour, with a black center or without a black center. H_2S (–) colonies are colourless or light pink with darker centers, and lactose (+) colonies are yellow or without the characteristic blackening.

Mannitol salt agar (MSA) is both a selective and differential media used for the isolation of Staphylococci from mixed cultures. This medium uses the advantage of high tolerance of staphylococci to salinity, to use sodium chloride as a selective agent, since only the staphylococci and halophilic enterobacteria are able to grow freely at this concentration of salt employed in this medium while other bacteria are inhibited. It also exploits the correlation between the pathogenic and fermentative capacity of mannitol of staphylococci, to establish a presumptive diagnosis. Mannitol fermentation with an accumulation of acid products is shown by the phenol red indicator turning yellow that produces a yellow halo surrounding the presumptive pathogen colonies, meanwhile the rest of the medium remains orange in colour. The typical appearance of the colonies after the correct incubation is as follows: Presumptive pathogenic staphylococci (coagulase +) is mannitol positive and are big colonies with a yellow halo. Non-pathogenic Staphylococci (coagulase -) are usually mannitol negative and are small colonies without halo or change in colour.

Kligler Iron Agar (KIA) Solid and differential medium for primary identification of enterobacteria based on the fermentation of two sugars and the hydrogen sulphide production according ISO Standard 6340. In this medium, lactose fermentation and H_2S production can be detected, so it allows a presumptive diagnostic of most enterobacteria. Glucose fermentation is shown by acid production, which makes the indicator turn from red to yellow, but since there is

little sugar (dextrose), acid production is very limited and then a reoxidation of the indicator is produced on the surface of the medium, and the indicator remains red. Otherwise, when lactose is fermented, the large amount of acid produced avoids reoxidation and then all the medium turns to yellow. Hydrogen sulphide production is indicated by the medium turning black, due to the reaction of H_2S (liberated from tiosulfate) with the Fe ions from ammonium iron citrate.

MacConkey Sorbitol Agar is Selective and differential solid medium for the detection of Enterohaemorrhagic Escherichia coli (EHEC O157: H7) according ISO standard. The only modification on the typical MacConkey Media formulations is the replacement of lactose with sorbitol. The enterohaemorrhagic strains do not use this substrate and produce colorless colonies, but the other serotypes can ferment sorbitol and produce red colonies. In all others aspects, MacConkey Agar with sorbitol works similarly as the other media in the MacConkey group. Peptone supplies the nitrogen and sodium chloride the osmotic environment. Crystal violet and bile salts inhibits the growth of gram positive bacteria. Neutral red acts as the pH indicator.

Mueller Hinton Agar has proved to be one of the most efficient medium in the anti-bacterial susceptibility testing. Without the addition of blood it can even be used for sulfonamide sensitivity testing since it is free from most of its antagonists (nucleotides, etc.). If this type of assay is conducted, the zones of inhibition should be examined just after 12-18 hours, before the usual overgrowth occurs, since after 24 hours it tends to interfere with the examination of sulfonamides sensitivity. For this purpose, a small inoculum will help the early formation of zones of inhibition. It should amount to a 100 to 300 times smaller inoculum than that of the corresponding strain which is used in the antibiotic sensitivity testing.

14.2 Common Biochemical test

1 Indole Test

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophan to indole. Certain bacteria are able to oxidize the amino acid, tryptophan, with tryptophanase enzymes to form three indolic metabolites - indole, skatole (methyl indole), and indoleacetate. Indole, when split from the tryptophan molecule, can be detected with the addition of Kovac's reagent.

Method

Inoculate nutrient broth with one loop-full of culture and incubate at 37^{o}C for 24 hrs. Then add 5 drops of Kovác's reagent directly to the tube.

A positive indole test is indicated by the formation of a pink to red color (“cherry red ring”) in the reagent layer on top of the medium within seconds of adding the reagent.

If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

2 H_2S production test

The activity of some bacteria on sulfur containing amino acids frequently results in the liberation of H_2S. The H_2S is usually tested for by demonstrating its ability to form black lead salt.

Method

Inoculate a loop-full of culture in 2% peptone water. Insert a lead acetate paper and incubate at 37°C for 24 hrs. If H_2S is produced, the blackening of lead acetate paper will take place.

3 Urease test

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urease-positive bacteria from other Enterobacteriaceae.

4 Methyl red test

The methyl red test is employed to detect the production of sufficient acid during fermentation of glucose and the maintenance of acid condition such that the pH of an old culture is sustained below a value of about 4.5.

Method:

Inoculate glucose phosphate broth with test culture and incubate at 37°C for 24 hr. Add about five drops of methyl red indicator solution. A distinct red colour is considered to be a positive test and yellow is negative.

5 Voges- Proskauer’s test

This is a test for the production of acetyl methyl carbinol from glucose. To the inoculated medium after incubation, alkali is added, in the presence of which any acetyl methyl carbinol present becomes oxidized to diacetyl. The diacetyl will combine with creatine to give a red colour.

Method

Inoculate glucose phosphate broth with test culture and incubate for 24hr at 37 °C. Pour ¼ th of the culture into a clean test tube. Add 0.5 ml (8- 10 drops) of the L-naphthol solution and 0.5 ml of the 40% KOH solution containing 0.3% creatine. Shake thoroughly and allow to stand for 5 to 30 minutes. The appearance of a pink to red color indicates the presence of acetyl methyl carbinol.

6 Citrate utilization test

The citrate test screens the ability of an organism to utilize citrate as the sole carbon and energy source for growth. A positive diagnostic test rests on the generation of alkaline by-products of citrate metabolism. The subsequent increase in the pH of the medium is demonstrated by the color change of a pH indicator.

Method

Fresh (16- to 18-hour) pure culture lightly streaks the surface of the slant with a wire needle and Incubate at 37^{o}C for 24 hrs. A needle is the preferred sampling tool in order to limit the amount of cell material transferred to the agar slant. Citrate utilization requires oxygen and thus screw caps, if used, should be placed loosely on the tube.

Citrate positive: growth will be visible on the slant surface and the medium will be an intense Prussian blue. The alkaline carbonates and bicarbonates produced as by-products of citrate catabolism raise the pH of the medium to above 7.6, causing the bromothymol blue to change from the original green color to blue.

Citrate negative: trace or no growth will be visible. No color change will occur; the medium will remain the deep forest green color of the un inoculated agar. Only bacteria that can utilize citrate as the sole carbon and energy source will be able to grow on the Simmons citrate medium, thus a citrate-negative test culture will be virtually indistinguishable from an un inoculated slant.

7 Catalase test

This test is primarily used to differentiate catalase producing bacteria, (eg. *Staphylococci*) from those non-catalase producing bacteria (*Streptococci*)

Principle

The enzyme catalase is present in most cytochrome-containing aerobic and facultative anaerobic bacteria. Catalase is the enzyme which has one of the highest turnover numbers compared to all other enzymes; one molecule of catalase has the ability to convert millions of molecules of hydrogen peroxide to water and oxygen in each second. Catalase production and activity can be detected either by adding the substrate H_2O_2 to an appropriately incubated (18 to 24 hours) tryptic soy agar slant culture or by slide test. Organisms which produce the Enzyme break down the hydrogen peroxide and the resulting O_2 production produces bubbles in the reagent drop, indicating a positive test. Organisms lacking the cytochrome system also lack the catalase enzyme and are unable to break down hydrogen peroxide, into O2 and water and are catalase negative.

Required

3% H_2O_2, wire loop, slide & test bacteria

Method

1. Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using wire loop
2. Place a drop of 3% H_2O_2 on to the slide and mix
3. look for immediate bubbling

Result

Active bubbling--------positive test catalase producing bacteria

No active bubbling---------negative test non catalase producing bacteria

8 Coagulase

It is used to differentiate coagulase producing bacteria from those non coagulase producing bacteria. The coagulase test is used to distinguish between pathogenic and nonpathogenic members of the genus Staphylococcus. S. *aureus* strains are capable of coagulating plasma in the tube test and will produce clumps of cells in the slide test. All pathogenic strains of S. aureus are coagulase positive whereas the nonpathogenic species (S. epidermidis) are coagulase negative.

Principle

The enzyme coagulase causes plasma to clot by converting fibrinogen to fibrin. Most strains of S.aureus produce one or two types of coagulase; free coagulase and bound coagulase. Bound coagulase is localized on the surface of the cell wall and reacts with α- and β-chains of the

plasma fibrinogens to form a coagulate. Free coagulase is an enzyme that is secreted extracellularly which reacts with prothrombin and its derivatives. The coagulase test can be performed using two different procedures - Slide test and tube test. The slide test is simple, giving results within 10 seconds, but it can give false negatives. The tube test is the definitive test, however, it can take up to 24 hours to complete. Free coagulase can be detected in tube coagulase test and bound coagulase can be detected in slide coagulase test.

Required

Rabbit or human plasma, slides, test tubes, wire loops, burner & test bacteria

Detection of Bound Coagulase - Slide Test

This method measures bound coagulase. The bound coagulase is also known as clumping factor. It cross-links α and β chain of fibrinogen in plasma to form fibrin clots that deposit on the cell wall. As a result, individual cocci stick to each other and clumping is observed.

1. Divide the slide into two sections with grease pencil. One should be labeled as „test" and the other as „control".
2. Place a small drop of distilled water or normal saline on each area.
3. Emulsify one or two colonies of Staphylococcus on MSA plate on each drop to make a smooth suspension.
4. The test suspension is treated with a drop of rabbit plasma and mixed well with a needle or applicator sticks.
5. Do not put anything in the other drop that serves as control. The control suspension serves to rule out false positivity due to auto agglutination.
6. Clumping of cocci within 5-10 seconds is taken as positive

Some strains of S.aureus may not produce bound coagulase, and such strains must be identified by tube coagulase test.

Detection of Free Coagulase - Tube Coagulase Test

This method helps to measure free coagulase. The free coagulase secreted by S.aureus reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma.

1. Three test tubes are taken and labeled "test", "negative control" and "positive control".
2. Each tube is filled with 1 ml of 1 in 10 diluted rabbit plasma.
3. To the tube labeled as test, add 0.2 ml of overnight broth culture of test bacteria.

4. To the tube labeled as positive control, add 0.2 ml of overnight broth culture of known S. aureus.

5. To the tube labeled as negative control, add 0.2 ml of sterile broth.

6. All the tubes are incubated at 37°C.

7. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube.

8. If the test remains negative until four hours at 37°C, the tube is kept at room temperature for overnight incubation.

Samples must be observed for clotting within 24 hours. This is because some strains that produce coagulase also produce an enzyme called fibrinolysin, which can dissolve the clot. Therefore, the absence of a clot after 24 hours is no guarantee that a clot never formed

15 Annex III

15.1 Media Formulations and Directions

Urea Agar Base

Table 10 Ingredient composition of Urea Agar Base (g/L)

Pancreatic digest of gelatin	1 g
NaCl	5g
Dextrose	1 g
KH_2PO_4	2 g
Phenol red	0.012 g
Agar	15g
Water	900ml

Dissolve 29g of the powder in 100ml of purified water. Mix thoroughly. Sterilize by filtration. Dissolve 15g of agar in 900ml of purified water. Autoclaving at 1210c for 15min. cool to 500c and 100ml of urea agar base. Mix thoroughly and dispense aseptically in sterile tube. Cool tube medium in slanted position so that deep butts are formed. Do not remelt the complete medium. Test samples of the finished product for performance, using stable, typical control culture. Final pH 6.8+0.2.

Kligler Iron Agar

Table 11 Ingredient composition of Kligler Iron Agar (g/L)

Polypeptone peptone	20 g
Lactose	20 g
Dextrose	1 g
NaCl	5 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.5 g
Agar	15 g
Phenol red	0.025g
Distilled water	1 liter

Dispense 49g in 1L of deionized water. Soak for 10min, swirl to mix and bring to the boil. Dispense in to tubes and sterilize by autoclaving for 15min at 1210c. Let the medium set as a slope ensuring that the slant is over a butt about 3cm deep.

Lysine Iron Agar

Table 12 Ingredient composition of Lysine Iron Agar (g/L)

Gelysate or peptone	5 g
Yeast extract	3 g
Glucose	1 g
L-Lysine hydrochloride	10 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate (anhydrous)	0.04g
Bromcresol purple	0.02g
Agar	15 g
Distilled water	1 liter

Suspend 24.2g of the powder in 1L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1min to completely dissolve the powder. Autoclave at 1210c for 15 min. cool tubed medium in a slant position for slant. Test samples of the finished product for performance using stable, typical control culture

MacConkey Agar

Table 13 Ingredient composition of MacConkey Agar (g/L)

Proteose peptone or polypeptone	3g
Peptone or gelysate	17g
Lactose	10g
Bile salts No. 3 or bile salts mixture	1,5g
Neutral red	0.03g
Crystal violet	0.001g
NaCl	13.5g
Distilled water	1L

Suspend 52g in 1L of distilled water .bring to the boil to dissolve completely. Sterilize by autoclaving at 1210c for 15min dry surface of the gel before inoculation.

Mannitol Salt Agar

Table 14 Ingredient composition of Mannitol Salt Agar (g/L)

Beef extract	1g
Polypeptone	10g
NaCl	75g
Mannitol	10g
Phenol red	0.025g
Agar	15g
Distilled water	1L

Suspend 111 g of powder in 1 L of distilled water and bring to the boil. Dispense in tubes or flasks and sterilize by autoclaving at 121°C for 15 minutes.

Motility Test Medium (Semisolid)

Table 15 Ingredient composition of Motility Test (g/L)

Beef extract	3g
Peptone or gelysate	10g
*NaCl	5g
Agar	4g
Distilled water	1L

Suspend 20g in 1L distilled water heat to boiling to dissolve the medium completely. Dispense in tube and sterilize by autoclaving at 15lbs pressure (121^0c) for 15min. allow the tube medium to cool in an upright position.

Mueller-Hinton Agar

Table 16 Ingredient composition of Mueller-Hinton Agar (g/L)

Beef, infusion from	300g
Acidicase Peptone (BBL) Or Casamino Acids (Difco)	17.5g
Starch	1.5g
Agar	17g
Distilled water	1L

Add 21 g of powder to 1 L of distilled water and dissolve it completely. Distribute in suitable containers. Sterilize by autoclaving at 121°C for 15 minutes. PH, 7.3 ± 0.2.

Nutrient Broth

Table 17 Ingredient composition of Nutrient Broth (g/L)

Beef extract	3g
Peptone	5g
Distilled water 1 liter	1L

Dispense 20g in 1000ml distilled water. Heat if necessary to dissolve the medium completely. Dispense in 50ml amount. Sterilize by autoclaving at 15 lbs pressure (121^0c) for 15min. Final pH, 6.8 ± 0.2.

Rappaport-Vassiliadis Medium

Table 18 Ingredient composition of Rappaport-Vassiliadis Medium (g/L)

Tryptone	5g
NaCl	8g
2PO4	1.6g
Distilled water	1L

Dissolve 26.8 g of powder in 1 L of distilled water, heating if necessary to help dissolve the powder. Dispense into test tubes or flasks and sterilize by autoclaving at 121°C for 15 minutes.

Simmons Citrate Agar

Table 19 Ingredient composition of Simmons Citrate Agar (g/L)

Sodium citrate	2g
NaCl	5g
K_2HPO_4	1g
$NH_4H_2PO_4$	1g
$MgSO_4$	0.02g
Bromthymol blue	0.08g
Agar	15g
Distilled water	1L

Suspend 24.2g of the powder in 1L of purified water. Mix thoroughly Heat with frequent agitation and boil for 1min to completely dissolve the powder. Autoclave at 1210c for 15 min. cool tubed medium in a slant position for slant. Test samples of the finished product for performance using stable, typical control culture

Sorbitol-MacConkey Agar

Table 20 Ingredient composition of Sorbitol-MacConkey Agar (g/L)

Peptone or gelysate	17g
Protease peptone No. 3 or polypeptone	3g
Sorbitol	10g
Bile salts, purified	1.5g
NaCl	5g
Agar	13.5g
Neutral red	0.03g
Crystal violet	0.001g
Distilled water	1L

Suspend 50g of the powder in 1L of purified water. Mix thoroughly Heat with frequent agitation and boil for 1min to completely dissolve the powder. Autoclave at 1210c for 15 min. cool tubed medium in a slant position for slant. Test samples of the finished product for performance using stable, typical control culture.

Trypticase (Tryptic) Soy Agar

Table 21 Ingredient composition of Trypticase (Tryptic) Soy Agar (g/L)

Trypticase peptone	15g
Phytone peptone	5g
NaCl	5g
Agar	15g
Distilled water	1L

Suspend 40g in 1L distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (121^0c) for 15 min. Final pH, 7.3 ± 0.2

Xylose Lysine Deoxycholate (XLD) Agar

Table 22 Ingredient composition of Xylose Lysine Deoxycholate (XLD) Agar (g/L)

Yeast extract	3g	Ferric ammonium citrate	0.8g
L-lysine	5g	Sodium thiosulfate	6.8g
Xylose	3.75g	NaCl	5g
Lactose	7.5g	Agar	15g
Sucrose	7.5g	Phenol red	0.08g
Sodium desoxycholate	2.5g	Distilled water	1L

Heat with agitation just until medium boils. Do not overheat. Pour into plates when medium has cooled to 50°C. Let dry about 2 hr with covers partially removed. Then close plates. Final pH,7.4 i 0.2. Do not store more than 1 day.

Declaration

I, the undersigned, declare that this M.Sc. thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the thesis have been duly acknowledged.

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Signature:_____

Date of submission:_____

This thesis has been submitted with our approval as advisors.

Advisor: Kassu Desta (MSc, PhD fellow)

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.