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ANTIMICROBIAL RESISTANCE TEST OF SALMONELLA ISOLATES FROM MILK AND FECAL SAMPLES OF APPARENTLY HEALTHY DAIRY CATTLE IN DEBREZEIT

MSC THESIS

BY

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Msc Program in Veterinary Microbiology

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College of Veterinary Medicine and Agriculture, Bishoftu

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Veterinary Microbiolgy

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October, 2014

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And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of veterinary microbiology.

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DEDICATION

This thesis manuscript is dedicated to Dr. Mulugeta Hawas a renowned medical doctor and loving father who has given his time, love money and life for my education. I wish you could have seen this dad, rest in peace.

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STATEMENT OF AUTHOR

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

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LIST OF ABBREVIATIONS

ACSSUT	Ampicillin, Chloramphenicol, Streptomycin, Sulfonamides, and Tetracycline
AMC	Amoxicillin
ART	Acid Tolerance Response
BAM	Bacteriological Analytical Manual
BGA	Brilliant Green agar
BGS	Brilliant Green sulfadiazine or sulfapyridine
BIS	Bismuth Sulphite agar
BPW	Buffered Peptone Water
C	Chloramphenicol
CBDS	Cell-Wall-Binding Domains
CDC	Centers for Disease Control and Prevention
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamycin
CSA	Central Statistics Agency
DNA	Deoxyribo Nuclic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration

LIST OF ABBREVIATIONS CONTINUED

FOX	Cefoxitin
FSIS	Food Safety and Inspection Service
FTI	Flow Through Immuno-capture
GI	Gastro Intestine
H_2S	Hydrogen Sulphide
HRP	Horse Radish Peroxidase
IMS	Immune-Magnetic Separation
ISO	International Organization for Standardization
K	Kanamycin
KW	Kauffman-White
LAC	Lactose
MDR	Multi Drug Resistance
MKTTN	Muller-Kauffmann Tetrathionate-Novobiocin
MLST	Multi-Locus Sequence Typing
MLVA	Multiple-Locus Variable Number Tandem Repeat Analysis
MR	Methyl Red

LIST OF ABBREVIATIONS CONTINUED

Na	Nalidexic Acid
NCCLS	National Committee For Clinical Laboratory Standards
NSAID	Non-Steroidal Anti Inflammatory Drug
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
QRDR	Quinolone Resistance Determining Region
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase
RV	Rapaport Vassilide
S	Streptomycin
SXT	Cotrimoxazol
TE	Tetracycline
TSA	Trip Soya Agar
TSI	Triple Sugar Iron
TT	Tetrationate
USDA	United States Department of Agriculture
VIDAS	Vitek Immuno Diagnostic Assay System
VP	Voges Proskauer
W	Trimetoprim
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

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ANTIMICROBIAL RESISTANCE TEST OF SALMONELLA ISOLATES FROM MILK AND FECAL SAMPLES OF APPARENTLY HEALTHY DAIRY CATTLE IN DEBREZEIT

By

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ABSTRACT

A cross sectional study to determine the antimicrobial profile of salmonella isolated from milk and fecal samples of apparently healthy cattle found in dairy farms of Debrezeit was undertaken from November 2013- April 2014. A total of 296 samples (148 fecal and milk each) were collected and processed in college of veterinary medicine and agriculture debrezeit. The overall prevalence of salmonella was found to be 12.8%. Prevalence of 2.0 and 10.8% was observed from milk and fecal samples respectively. The isolates were tested for the effect of 11 antimicrobial by disk diffusion technique they have indicated 89.5%, 100%, 42.1%, 63.3%, 15.8%, 10.5%, 31.6%,42.1%, 52.6% resistant to amoxacyline, tetracycline and gentamycin, kanamycin, trimetoprim, streptomycin, cotrimoxazol, chloramphenicol, nalidixic acid and cefoxitin respectively no resistance has been found for ciprofloxacin. However no statistical difference ($P>0.05$) was observed between milk and fecal samples. Totally 8 Multiple Drug Resistance (MDR) pattern were also observed. The highest MDR was for three antibiotics with the combination Amc, Te, Cn being more frequent. In general MDR to three, seven and eight antibiotics dominate the resistance patterns (31.6%, and 21.1% each). From this study we conclude that milk can be a potential source of drug resistant salmonella infection and multidrug resistance is developing due to unmonitored and inappropriate use of drugs in dairy farm which needs due consideration as it affects the dairy industry and the people at large.

Key words: *Salmonella*, Prevalence, Milk, Feces, Antimicrobial resistance, Debrezeit

1. INTRODUCTION

Ethiopia maintains the largest cattle population in Africa at 50.8 million, of these, 7.4 million cows whose primary purpose is milk production and 10.7 million are milking cows used for milk regardless of their primary purpose (CSA, 2009). Milk and dairy products play a significant role in the nutrition and health of the people. Animal products accounts for 8% of the total food consumption in Ethiopia and approximately 50% of this is accounted for by milk and dairy products. The dairy industry in Ethiopia has grown significantly in the past few decades and possesses the potential to expand greater in the future (Dailey, 2011).

Even though there has been a large and consistent increase in production, the dairy industry has generally not been able to keep up with the rapidly expanding population with a 3.0% per year growth and the demand for milk products is expected to increase considerably with a large portion coming from urban consumers (Dailey, 2011).

The low average yield is in large part due to low numbers of exotic dairy cattle and crossbreeds, limited focus on genetic improvement, lack of selection criteria for bulls, lack of pedigree information and low efficiency and effectiveness of AI technicians, lack of adequate veterinary service which leads to poor health of dairy cattle which can affect productivity through death, weight loss and poor fertility it also lowers milk quality and introduce pathogens into the milk (Redda, 2002).

Milk-borne illnesses have been recognized since the beginning of the dairy industry. Milk is a highly nutritious food that is ideally suited for growth of pathogenic and spoilage organisms. Dairy cattle are believed to be commonly exposed to *Salmonella* spp. through feed, water, wild birds, rodents, and persistently contaminated environments (Wells, *et al.*, 2001). Infected cattle may shed *Salmonella* species for longer period of time and may become chronic carriers. Farms may be persistently infected for years by the continuous cycle of environmental contamination, cattle infection and fecal shedding. *Salmonella* may survive in fresh water systems for 56 days or more, depending on conditions (Murray, 2000) and there is concern that contamination of aquifers and surface waters through cattle waste may occur. Direct contamination of surface water, leakage or overflow from lagoons, milking shed waste water disposal and run off from pastures or barnyards can all contribute to local water contamination. The contaminated water may then be a reservoir for infection of humans, cattle or other species (Graham, 2003).

Salmonellae are disease causing organisms common in cattle. They are often concern due to disease of cattle and the potential to infect human that come in contact with cattle or consume dairy product or bovine meat product. It is a leading cause of food borne illness (White *et al*., 2001). Globally, more than 93million cases of gastroenteritis are caused by non-typhoidal *Salmonella* with 155,000 deaths each year. Of these cases, 80.3% were estimated to be food borne. Salmonellosis, the diseases caused by bacteria of the genus *Salmonella*, is a common intestinal illness caused by numerous *Salmonella* serovars with clinical manifestations that vary from severe enteric fever to mild food poisoning both in animals (Radostits *et al*., 2007) and humans (Hohmann, 2001).

Various serotypes of *Salmonella* are well established as a cause of disease in both juvenile and adult dairy cattle. The most common clinical signs are fever and diarrhea. Abortion due to bacteremia or endotoxemia can occur. In adult dairy cattle, clinical signs often occur during periods of reduced immune function such as the peri-parturient period. The risk period for calves varies depending upon serotype (usually <14 days of

age for *S.* Typhimurium, 1 week to 6 months of age for *S. dublin*). The disease is spread through fecal-oral transmission and is maintained within cattle populations by carrier animals, infected calves, environmental contamination (Smith, 1990).

During early sixties, *Salmonella* resistance to single antibiotic was reported and since then multiple drug resistance (MDR) has been reported worldwide (Threlfall *et al.*, 1997). Current global scenario has showed that an increased number of antibiotic resistant *Salmonella* spp. from humans and farm animals. This resulted into major public health concern that *Salmonella* spp. could become resistant to antibiotics used in human medicine, thus, reducing therapeutic options and threatening the lives of infected individuals. The uncontrolled use of antibiotics in farm animals and aquaculture system has contributed tremendously to the emergence and persistence of resistant strains (Young, 1994). A study carried out for antibiotic resistance pattern in *Salmonella* isolated from swine by Gebreyes *et al*., (2000) demonstrated that a total of 49% *Salmonella* strains exhibited MDR pattern. Multidrug-resistant phenotypes have been increasingly described among *Salmonella* species worldwide according to the WHO report on infectious disease. The widespread use of fluoroquinolone is in practice due to broad spectrum activity, high efficiency, and various applications in human and veterinary medicine (WHO, 2000).

In Ethiopia, despite attempts to study prevalence of *Salmonella* mainly in poultry and beef, the status in milk and milk products is still insufficient. However, studies made elsewhere indicated that milk and milk products are important source of *Salmonella* particularly among those raw consumers (WHO, 2000). Ubiquitous nature of *Salmonella*, unhygienic condition prevailing at the farm levels and food handlers, and habit of consuming milk and milk products in raw suggest that milk and milk products can act as source of *Salmonella* organisms in Ethiopia. Considerable proportion of them might have developed resistance to antimicrobials that are commonly used in both the veterinary and public health (Tesfaw *et al*., 2013). This problem might have a significant

toll on towns like Debre-Zeit which are becoming home of the largest milk and milk product supplier industries to Addis Ababa and its surroundings.

GENERAL OBJECTIVE:-

- To isolate and determine the antimicrobial resistance profile of salmonella isolates from milk and fecal samples of apparently healthy dairy cattle in Debre-Zeit.

SPCIFIC OBJECTIVES:-

- To isolate salmonella from dairy cattle milk and feces by using different microbiological diagnostic techniques.
- To determine the antibiotic susceptibility of salmonella for different antibiotics.
- To make comparisons between fecal and milk samples isolated Salmonella antibiotic susceptibility.

2. LEATRATURE REVIEW

2.1. *Salmonella*

*Salmonella*e belong to the family Enterobacteriaceae, and are facultative anaerobic gram-negative straight rods of 0.7 - 1.5 x 2.0 - 5.0 μm in size and shape. *Salmonellae* are facultative anaerobe, Gram-negative rods belonging to the family Enterobacteriaceae. Although members of this genus are motile by peritrichuous flagella, non-flagellated variants, such as *Salmonella* Pullorum and *Salmonella* Gallinarum and non-motile strains resulting from dysfunctional flagella do occur (D'Aoust, 1997). The colonies are generally 2-4 mm in diameter. *Salmonella*e reduce nitrates to nitrites and usually produce gas from glucose and hydrogen sulfideon from triple-sugar iron agar. They are indole-negative, urease-negative and usually utilize citrate as a sole carbon source (Brenner, 1984).

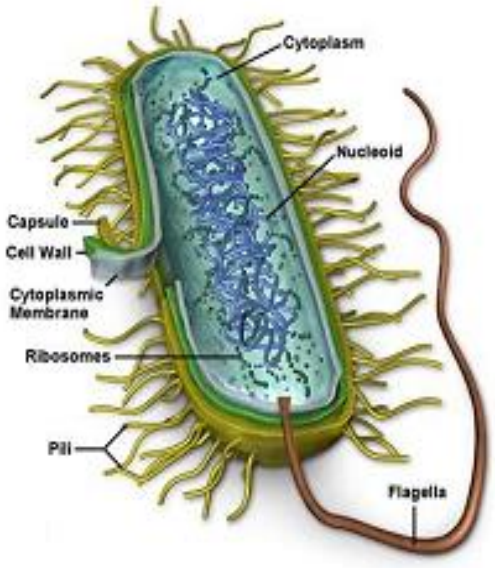

Fig.1. Morphology of flagellated *Salmonella* (Haque, 2005)

2.1.1. Classification and Nomenclature of Salmonella

Salmonella nomenclature has changed many times and still is not stable. The genus *Salmonella* was previously differentiated into two species: *Salmonella enterica* and *Salmonella bongori*. However, a new species, *S. subterranea* was identified and validated (Shelobolina *et al*., 2004; Validation List No: 102, 2005). Among them, the species *Salmonella enterica* (*S. enterica*) is further divided into the six subspecies *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *Salamae* (II), *S. enterica* subsp. *Arizonae* (IIIa), *S. enterica* subsp. *Diarizonae* (IIIb), *S. enterica* subsp. *Houtenae* (IV), and *S. enterica* subsp. *indica* (VI). Formerly, *S. bongori* was the subspecies V, but later considered as a separate species (Tindall *et al*., 2005).

To avoid confusion between serovars and species, the serovar name is not italicized and starts with a capital letter. When cited the first time in a report, the genus name is given followed by the word "serotype" (or the abbreviation "ser.") and then the serotype name, e.g., *Salmonella* serotype or ser. Choleraesuis. Afterward the name may be shortened with the genus name followed directly by the serotype name, e.g., *Salmonella* Choleraesuis or *S*. Choleraesuis (Popoff *et al*., 2002).Because the type species name, *enterica*, was not approved before 2005, serotype names are used directly after the genus name without mention of the species. Following official approval of "*enterica*" as the type species name, further amendment to include the species name in the *Salmonella* nomenclature of the CDC may be expected (Lin and Chang, 2007).

For those designated by their antigenic formulae, the subspecies name is written in Roman letters (not italicized) followed by their antigenic formulae, including O (somatic) antigens, H (flagellar) antigens (phase 1), and H antigens (phase 2, if present). A colon is used between each antigen, e.g. *Salmonella* serotype II 39:z10:z6. For serotypes in *S.*

bongori (which previously belonged to subgenus V), V is still used for consistency, e.g., S. V13,22:z (Popoff *et al*., 2002).

Table 1. Current Nomenclature of Salmonella

Taxonomic position (writing format) and nomenclature				
Genus (capitalized, italic)	Species (italic)	Subspecies (italic)	Serotypes/serovars (capitalized, not italic)	No. of serotypes in each species or subspecies
Salmonella	*enterica*	*enterica* (subspp. I)	Typhi, Typhimurium,…	1504
		salamae(subspp. II)	9,46:z:z39	502
		arizona(subspp. IIIa)	43P:z29:-	95
		diarizona(subspp.IIIb)	6,7:1,v:1,5,7	333
		houtenae (subspp. IV)	21:m,t:-	72
		indica (subspp.VI)	59:z36:-	13
	bongori	Subspp.V	13,22:z39:-	22
	subterranea			

Source: (Lin and Chang, 2007)

2.1.2. Physiology and Biochemical Characteristics

The biochemical properties of *Salmonella* spp. show that almost all *Salmonella* serovars do not produce indole, hydrolyze urea, and deaminate phenylalanine or tryptophan. Most of the serovars readily reduce nitrate to nitrite and most ferment a variety of carbohydrates with the production of acid, and reported to be negative for Voges-Proskauer (VP) reaction. The other prominent characteristics of *Salmonella* are that most serovars produce hydrogen sulfide (H_2S) and decarboxylate lysine, arginine and orinithine with few exceptions (e. g. *Salmonella enterica* subsp. *arizonae* and *Salmonella enteric* subsp *diarizonae*). Most of *Salmonellae* utilize citrate with a few exceptions such as *Salmonella* Typhi, *Salmonella* Paratyphi. A and a few *Salmonella* Choleraesuis serovars. Dulcitol is generally utilized by all serovars except *Salmonella enteric* subsp. *arizonae* (Illa) and *Salmonella enterica* subsp. *diarizonae* (lllb), whereas, lactose will not be utilized by most of the *Salmonella* serovars (Popoff and Le Minor, 2005).

Salmonellae are considered resilient microorganisms that readily adapt to extreme environmental conditions. *Salmonella* grow best at moderate temperature (35 -37°C), they can grow over a much wider temperature range, as low as 4 °C and as high as 48 °C. Thermal stress mutants of *Salmonella* Typhimurium has been reported to grow at elevated temperature of 54°C and some other serovars exhibited psychotropic properties by their ability to grow in foods stored at 2^o to 4°C (Droffner and Yamamoto,1992).

The physiological adaptability of *Salmonella* spp. was demonstrated by their ability to proliferate at pH values rangingfrom 4.5 to 9.5. Leyer and Johnson (1992) demonstrated the increased survival of acid-adapted *Salmonella* in fermented milk and refrigerated temperature.

Salmonellae have several virulence factors that contribute to causing diarrhea, bacteremia, and septicemia. These factors include the lipopolysaccharide of the outer wall, pili, flagella, cytotoxin, and enterotoxin (Murray, 1986).

2.1.3. Epidemiology

The most important infection route in animals is the fecal-oral. Respiratory infection is also possible; *Salmonella* may be spread by aerosols with the use of pressure hoses when cleaning stalls. Contaminated milk may also cause infections. Occasionally cattle may excrete *Salmonella* in their milk, but more frequently the milk is contaminated by infected feces during the milking process (Gillespie and Timoney 1981).

Newborn calves are exposed to *Salmonella* infection as the carrier cows shed the organism at parturition. The transmission of infection from one farm to another is mainly achieved by the purchase of infected cattle (Wray and Davies, 2000).

Non-symptomatic ruminants shed the bacteria intermittently and therefore infection is difficult to detect. Some asymptomatic carriers continue to eliminate *Salmonella*e in their feces for weeks, months or years after recovery from a clinical case: and by doing so these carriers contribute to the dissemination of Salmonellosis. Latent carriers are important in the epidemiology and persistence of *S.* Dublin on farms, because this bacterium can survive in the environment for over a year (Edrington *et al.,* 2004).

Cats, dogs, mice, rats, foxes and badgers have been shown to be infected with *S.* Typhimurium. Rats and mice that acquire *S.* Dublin infection do not play a major role in the spread of infection, but they might prolong the persistence of *Salmonella* on farms (Wray and Davies, 2000).

Another important infection source for animals is contaminated feedstuffs. Both formulated compound feeds and also vegetable proteins, such as soya, have been found to be contaminated with *Salmonella*. Imported animal protein, such as fishmeal, and waste

from the food industry, might also be contaminated. Compound-feed mills may be contaminated by *Salmonella* present in the ingredients, and the presence of *Salmonella* in cooling systems and storage bins may lead to the subsequent contamination of products both during and after processing. To minimize the risk for contamination of stored feed, effective rodent and bird control is important (Wray and Davies, 2000)

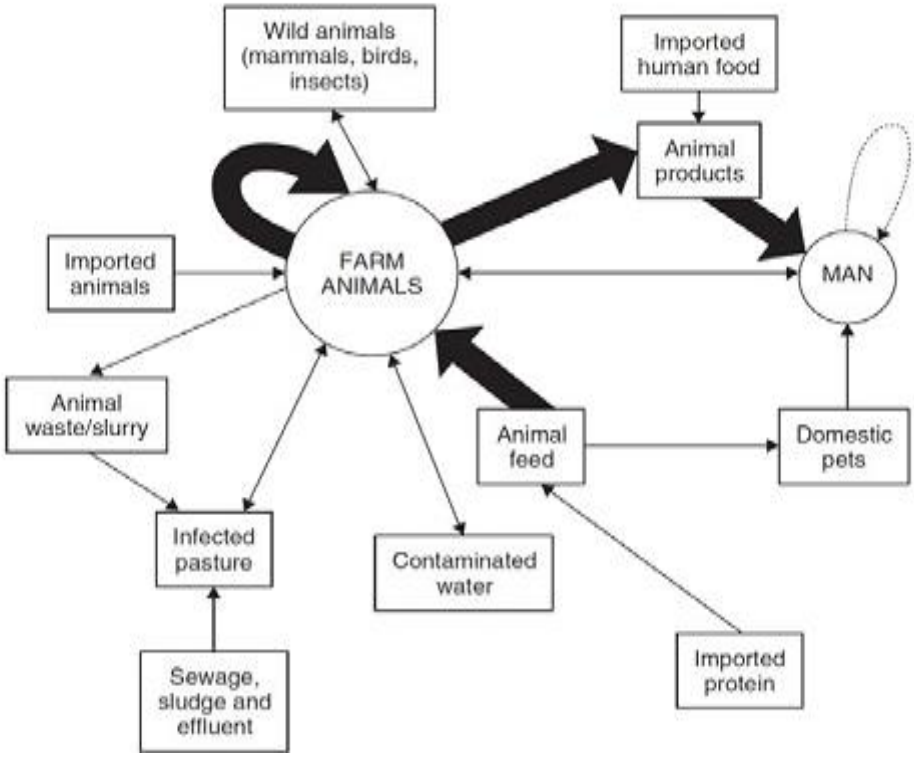

Fig.2. Route of transmission (Haque A, 2005)

2.1.4. Host Specificity

Host adaptation occurs in *Salmonella* at both the serotype level and at the level of phage type within a serotype. Clear associations have been demonstrated between *S. enteric* serovars Typhi, Paratyphi A, and Sendai and humans; *S.* Dublin and cattle; *S.* Choleraesuis and swine; *S.* Pullorum and Gallinarum and poultry; *S.* Abortusequi and horses; *S.* Abortusovis and sheep; and *S.* Arizona and reptiles. Interestingly, genomic

comparisons of *Salmonella* serovars showed that serovars Typhi, Paratyphi A, and Sendai clustered together, suggesting that they share genetic features (Chan *et al*., 2003).

S. Typhi appears to have an exclusive association with humans, but the other serotypes may cause disease in hosts other than those to which they have adapted. For example, even serotype Gallinarum may sometimes cause disease in mammalian host, and *S.* Dublin, *S.* Choleraesuis, and *S. arizona* are associated with disease in humans and other hosts to which the serotypes are not adapted. The vast majority of serotypes of *Salmonella enteric* show no host adaptation. *S.* Typhimurium and *S.* Enteritidis are frequently isolated from a variety of vertebrates with and without clinical disease and may be considered the least host-adapted serotypes. Typically, host-adapted serotypes cause severe systemic disease in adult as well as young hosts, whereas the unadapted serotypes are associated with enteric disease primarily in young hosts. It is interesting that *S.* Typhimurium, which causes a predominantly gastrointestinal illness in a wide range of hosts, causes typhoid like disease in mice. *S.* Abortusovis causes only a mild disease in adult sheep. Host adaptation may occur among subtypes of a serotype (Rabsch *et al*., 2002).

2.1.5. Clinical Features

The disease caused by *Salmonella*e is usually either systemic, or an acute enteritis. In the latter systemic disease is seen only in cases with decreased immune response. Animals are predisposed to clinical Salmonellosis by several stress factors, such as parasitism, viral infections, parturition, poor sanitation, poor nutrition, overcrowding, and transportation. Calves that have not received adequate colostrum within the first 12 hours of life are more susceptible to infection. Young animals are also less able to cope with dehydration (Barrow and Wallis, 2000).

The pathogenicity of *Salmonella* is both serovar- and host-dependent. Serovars such as Abortusovis and Pullorum/Gallinarum are adapted to sheep and poultry. Other serovars,

such as *dublin* and Choleraesuis, cause disease primarily in one animal species (cattle or pigs) but are opportunist pathogens of others (Barrow and Wallis, 2000).

Typical symptoms and signs in the acute phase of the infection are depression, anorexia, fever, weakness and diarrhea, which may be blood-stained. Pregnant animals may abort. Death occurs most frequently within a week from the onset of clinical signs; in calves often within a day or two. The mortality, usually in the range of 5 to 10 per cent, may be as high as 75 per cent. In calves that survive, there may later be signs of joint infection (Wray and Davies, 2000).

Animals can also be symptomless; they harbor the infection in their lymph nodes or tonsils without excreting the organism in their feces, but during stress these latent carriers may become active carriers or even clinical cases per se (Wray and Davies, 2000).

As a disease of adult cattle, Salmonellosis is often subclinical or characterized by mild diarrhea, lethargy and decreased food consumption. Even so, it may cause significant economic and production losses through reduced weight gain, feed efficiency, and milk product. In a recent study of risk factors for clinical disease associated with *S.* Typhimurium on Dutch dairies, Veling, *et al*., (2002b) noted that symptoms were seen only in adult cows on 66% of the affected farms.

Dairy cattle are believed to be commonly exposed to *Salmonella* spp. through feed, water, wild birds, rodents, and persistently contaminated environments but the epidemiology of this disease is not yet understood. Infected cattle may shed *S.* Typhimurium for up to 12 weeks after recovery, but rarely become chronic carriers. Farms, however, may be persistently infected for years by the continuous cycle of environmental contamination, cattle infection and fecal shedding. This chronic herd-level infection with subclinical individual animal illness and intermittent fecal shedding may pose a significant risk for humans through contamination of meat and milk products and of the environment. *Salmonella* may survive in fresh water systems for 56 days or more, depending on conditions and there is concern that contamination of aquifers and surface

waters through cattle waste may occur. Direct contamination of surface water, leakage or overflow from lagoons, milking shed waste water disposal and runoff from pastures or barnyards can all contribute to local water contamination (Huston, *et al*., 2002a).

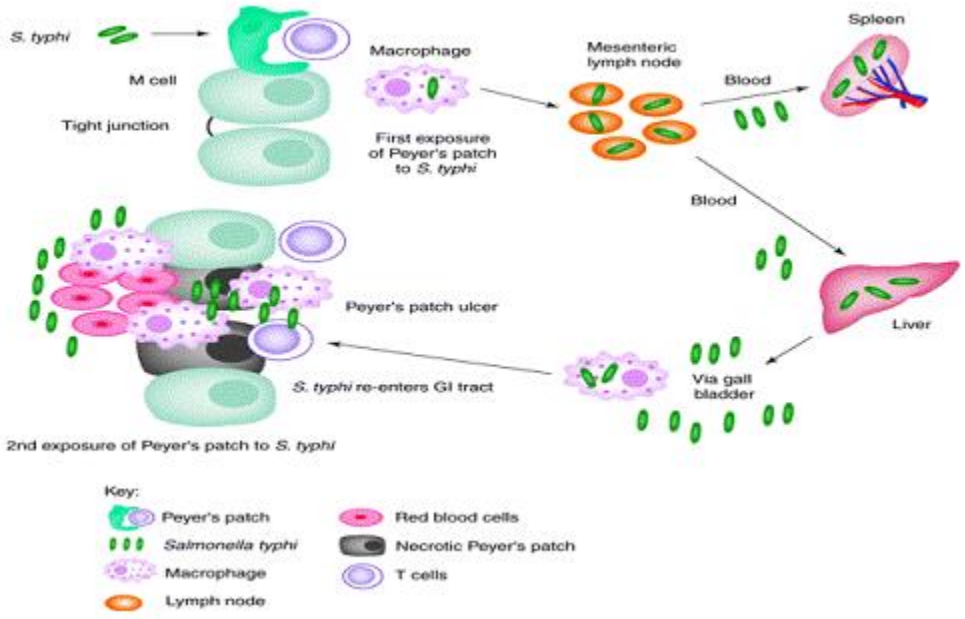

Fig.3. Pathogenesis of *S*.Typhi (Haque A, 2005)

2.1.6. Diagnosis

Generally, detection methods are based on physiological and biochemical markers of the organism. Cultural methods are based on nutrient acquisition, biochemical characteristics, and metabolic products unique to *Salmonella* spp. More rapid immunological and molecular screening methods of detection have been devised to detect cell surface markers and nucleic acids, respectively (Leminor and Popoff, 2001).

2.1.6.1. Culture methods

Culture based methods are still the most widely used detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. For instance, the US Food and Drug Administration (FDA) and the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA), requires an isolated organism as unambiguous proof of contamination (Alocilja and Radke, 2003).

Depending on the approach, standard culture methods typically require 5–7 days to obtain a result as they rely on the ability of *Salmonella* to multiply to visible colonies, which can then be characterized by performing additional biochemical and or serological tests. Due to their widespread use, numerous and varied bacteriological media (selective enrichment broths and selective agar plates) are applied to best monitor for *Salmonella* in food and food ingredients. The media may contain inhibitors in order to stop or delay the growth of non-target organisms, or particular substrates that only the target bacteria can degrade, or that confer a particular color to the growing colonies (Manafi, 2000).

Cultural methods typically involve the enrichment of a portion of the sample to recover sub-lethally injured cells due to heat, cold, acid, or osmotic shock in a non-selective pre-enrichment media, such as Buffered Peptone Water (BPW), and to increase the number of target cells as these are generally not uniformly distributed in foods, typically occur in low numbers, and may be present in a mixed microbial population. Next, primary enrichment cultures are typically inoculated into secondary selective enrichment broths, such as Selenite Cystine broth (SC), Rappaport Vasiliadis Soy broth (RVS), Tetrathionate Broth (TT), or Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn) and incubated at elevated temperatures (37°C or 42°C for 18-24 hours) before being struck onto selective agars such as Xylose Lysine Deoxycholate agar (XLD agar), Bismuth Sulphite agar (BIS), Brilliant Green agar (BG) with or without the addition of

sulfadiazine or sulfapyridine (BGS), modified semisolid Rappaport Vasiliadis(MSRV), *Salmonella* Shigella Agar, or Hektoen Enteric agar (Sandel *et al*., 2003; Gracias and McKillip, 2004)

Typical *Salmonella* colonies based on morphology and or indicative biochemical reactions on selective agars are then cultured onto non-selective media prior to confirmatory testing. There are well established confirmations and identification procedures for *Salmonella*. Preliminary identification is traditionally performed using classical biochemical and serological tests. Key biochemical tests include the fermentation of glucose, negative urease reaction, lysine decarboxylase, negative indole test, H_2S production, and fermentation of dulcitol. Serological confirmation tests typically utilize polyvalent antisera for flagellar (H) and somatic (O) antigens. Isolates with a typical biochemical profile, which agglutinate with both H and O antisera are identified as *Salmonella* species. Where results are inconclusive, it may be necessary to perform additional biochemical tests. Positive isolates are often sent for further serotyping to identify the serovar using specific antisera as per the Kauffman-White (KW) typing scheme recognizing 46 O antigens, and 119 H antigens, thereby permitting the characterization of 2,541 serotypes. Serotyping is a useful epidemiological tool in identifying circulating serotypes and to characterize outbreaks. The antigenic formula of Le Minor and Popoff (2001) is a standard method for naming the serovars.

However, serotyping is normally undertaken at reference laboratories and is rarely performed in routine food or clinical laboratories. Reference laboratories are also able to further type isolates using techniques such a phage typing, antibiotic susceptibility, pulsed-field gel electrophoresis (PFGE), or other emerging genetic typing technologies such Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) and Multi-locus sequence typing (MLST) (Kruy *et al.*, 2011).

Although standard culture methods are excessively time consuming, there is potential for further improvements, and thus many attempts have been made to maximize their

efficiency by introducing new technologies, making reliability of detection more convenient, user friendly, as well as by reducing the costs of materials and labor. For example, biochemical confirmatory tests may be easily replaced by commercial identification kits such as the API 20E or other commercially available bacterial identification kits. The detection of sub-lethally damaged cells is of utmost importance as these may still pose a risk to human health and may lead to false negative results. Strategies for the recovery of injured bacteria are based on overlay methods such as Tryptic soy agar (TSA) over layed on XLD selective agar (Kang and Fung, 2000) and other approaches also include the development of single enrichment broths where multiple step enrichments are usually required (Baylis *et al*., 2000).

Other novel approaches include the addition of bacteriophages for the elimination of back ground micro-flora that may out-compete the target organism. For example, RapidChek® SELECTTM *Salmonella* (Strategic Diagnostics Inc.) employees a primary enrichment media supplemented with a bacteriophage cocktail as a selective agent, which reduces the level of background flora in high burden samples allowing *Salmonella* to grow with minimal competition. In addition, there is also the development of enrichment broths for the concurrent enrichment of pathogens thereby reducing laboratory workloads with respect to the preparation of sample homogenates since different enrichment broths would no longer be required, and multiple analyses could be performed from a single universal enrichment culture (Kim and Bhunia, 2008).

Amendments to media have also been performed such as the addition of novobiocin and cycloheximide to decrease fungal overgrowth (Ricke *et al*., 1998). Lastly, and perhaps the most important advancement is the use of chromogenic or fluorogenic substrates in selective agars, permitting identification to be performed directly on the isolation plate, thereby expediting or eliminating the use of subculture media or additional biochemical tests as these media provide highly specific reactions, and help reduce the workload for unnecessary examination of suspect colonies arising from poor specificity of conventional agars (Manafi, 2000).

A number of selective chromogenic agar media specifically designed for the differentiation of *Salmonella* colonies are commercially available with varying success of adoption by regulatory agencies such as: *Salmonella* SMS (AES Chemunex), BBL CHROMagar (CHROMagar), RAPID'Salmonella (Bio-Rad Laboratories, S.A.), chromID Salmonella (BioMerieux), Harlequin Salmonella ABC (Lab M), Oxoid Brilliance Salmonella Agar (Oxoid), and Rambach Agar (Merck), among others. It is evident that the multitude of options for isolation of *Salmonella* and the lack of interlaboratory consistency make *Salmonella* isolation one of the most variable procedures in laboratories with new media available every year, promising to be more sensitive, specific, and rapid (Hyatt and Weese, 2004).

2.1.6.2. Immunomagnetic separation

In an attempt to reduce the length of routine microbiological analysis and to minimize the problems associated with rapid detection systems such as interference from foods and food ingredients debris, background micro-organisms, and lack of sensitivity, there has been a lot of interest in the development of separation and concentration techniques prior to detection. Various techniques have been utilized for this purpose including: centrifugation, filtration and lectin-based biosorbents (Payne *et al.,* 1992).

However, the most successful of approaches for the separation and concentration of target organisms has been the use of immune-magnetic separation (IMS). The advantages of IMS are that it reduces the total analysis time and improves the sensitivity of detection. IMS is rapid, technically simple, and specific method for the isolation of the target organisms (Shaw *et al,*. 1998).

Paramagnetic particles are coated with antibodies specific to the target organism and added to a post enrichment culture. The target organism is captured onto the magnetic particles and the whole complex is then removed from the system by the application of a

magnetic field. Target organisms are thus removed from food debris and competing microorganisms, which may otherwise interfere with the detection system. If required, the isolated complex may be re-suspended in an enrichment broth so that cell numbers can be rapidly increased to improve the sensitivity of detection assays. In addition, IMS by design can be used in conjunction with other rapid detection methods, including ELISA, conductance microbiology, electro chemi-luminescence, and polymerase chain reaction (PCR) to further increase its analytical sensitivity (Sapanova *et al.*, 2000).

IMS is more sensitive than conventional culture methods and is able to reduce the total culture analysis time by one to two days. The most commonly used commercial IMS bead for the recovery of *Salmonella* from food samples is Dynabeads anti-*Salmonella* (Invitrogen) (Warren *et al.*, 2007).

2.1.6.3. Immunological based methods

d. Rapid agglutination assays

Several rapid latex agglutination assay tests are widely used for the rapid detection of *Salmonella*. These assays however, are primarily used as a confirmation screen for presumptive *Salmonella* colonies after culture isolation from selective agar plates, with further confirmation and identification work carried out on those organisms giving a positive latex reaction. An aliquot of a colony suspension or enrichment broth is simply mixed with the latex reagent and after a few minutes rotation, the results are clearly visible. If the test is negative, the latex remains in smooth suspension and retains its original color. A positive result is indicated by distinct color agglutination against an altered background. By reducing the number of samples requiring further confirmatory testing, these tests save time and resources and allow negative results to be reported at least 24 hours earlier than by conventional culture methods. However, depending on the antibodies used they may lack specificity due to non-specific agglutination of some organisms (Cheesbrough and Donnelly, 1996).

e. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) also known as an enzyme immunoassay (EIA), is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. In the context of *Salmonella* detection, a sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtitre plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "Sandwich" ELISA). After the antigen is immobilized, a detection antibody linked to an enzyme such as Horse Radish Peroxidase (HRP) is added, forming a complex with the antigen. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies thatare not specifically bound. After the final wash step, the plate is developed by adding anenzymatic substrate (ABTS or 3,3',5,5'-tetramethylbenzidine) to produce a visible signal(colorimetric or fluorescent product) due to the enzymatic cleavage of the substrate.Colorimetric equipment is used to measure the signal indicating colorimetric equipment indicating the presence of target antigen in the sample (Westerman *et al.*, 1997)

Currently, there are numerous ELISA plate based assay systems for the detection on *Salmonella: Salmonella* ELISA (BIO ART SA), TRANSIA® PLATE *Salmonella* Gold (BioControl), and RIDASCREEN® *Salmonella* ELISA (R-Biopharm AG). Some of these tests have the advantage of being able to process numerous samples at once in 96 well microtitre plates, and some such as the Tecra™ *Salmonella* Visual Immunoassay (3M), provide a visual indication of detection without the use of colorimetric equipment. In addition ELISA systems have been automated to facilitate routine laboratory testing such as the EIAFoss (Foss Electronics) and the Vitek Immuno Diagnostic Assay System (VIDAS) (BioMerieux). Nevertheless, ELISA methods are not without disadvantages, some of which include high limits of sensitivity of >105 cfu/ml variable cell surface

antigen production; cross reactivity, and changes to antigens due to acetylation and changing recognition by assay antibodies (Kim and Slauch, 1999).

f. Lateral flow immunoassays

Commercially available lateral flow immunoassays for the detection of *Salmonella* include: DuPont™ Lateral Flow System *Salmonella*, Singlepath *Salmonella* (Merck), Reveal® *Salmonella* lateral flow (Neogen), VIP Gold (BioControl), and RapidChek® SELECT (SDIX). Recently, serotype specific lateral flow immunoassays for the detection of *S.* Enteritidis have also been introduced to serve the egg and poultry industry such as RapidChek® SELECT *S.* Enteritids (SDIX) and Reveal *S.* Enteritidis (Neogen). In general, these types of immunoassays are ideally suited where a low testing throughput is expected. The implementation of these tests is beneficial in that they require low technical expertise, and minimal capital expenditure (Westerman *et al.,* 1997).

2.1.6.4. Molecular Methods

a. Real-time PCR

The development of novel chemistries and instrumentation platforms enabling detection of PCR products on a real-time basis has led to widespread adoption of real-time PCR as the method of choice for detection of *Salmonella*. This method combines amplification and detection stages of the process so that nucleic acid amplification is monitored and recorded continuously hence eliminating the need for post-amplification steps such as gel electrophoresis. Total time for an analysis for the detection of *Salmonella* species is normally 20 to 48 hours but can be as little as 12 hours depending on the food matrix, enrichment conditions, and instrument run time. The main advantage of these PCR systems over other methods is in time saving, both in the total time from sampling to result and in the technical time needed to set up and run the assay (Park *et al.,* 2006).

b. Multiplex PCR

In multiplex PCR (mPCR), generally, the 16S rRNA gene is the most common target as it is routinely used to establish phylogenetic distinctions among bacteria. However, other target genes are also considered in order to achieve a high specificity for example, Rajtak *et al*., (2011) developed a two step real-time mPCR assay for the rapid screening of 19 *Salmonella* serotypes frequently encountered in humans, animals, and animal-associated meat products within the European Union. Specific primers for serotype differentiation were designed to target the genes encoding either phase 1 and 2 flagellar antigens *fliC*and *fljB*or unique serotype-specific loci. In addition, the assay simultaneously screened for the presence of the ampicilin-amoxicillin, chloramphenicol-florfenicol, streptomycin-spectinomycin, sulfanomides, and tetracycline (ACSSuT)-type multidrug resistance pattern, indicated by the *floR*gene, and for the *Salmonella* virulence plasmid encoded by the svp operon in *S.* Typhimurium. The assay represents a more rapid and reliable method for identification of large numbers of serotypes than assays using phenotypic serotyping methods (Rossello-Mora and Amman, 2001).

Multiplex PCR is thus quite versatile and numerous other assays have been published for the rapid detection and characterization of specific *Salmonella* serotypes analogous to mPCR approaches used for the differentiation of multiple species belonging to single genera such as gastroenteritis causing thermo-tolerant Campylobacter species or for the differentiation of the major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c, and 4b) commonly implicated in food borne listeriosis (Woods *et al*., 2008).

Lastly, and perhaps the largest impact that mPCR may provide in a near future is in the rapid and simultaneous detection of *Salmonella* concurrently with other bacterial pathogens. For instance, Gilbert *et al.,* (2003) established a mPCR assay in order to detect Salmonella along with *Campylobacter jejuni*, and *E. coli* O157:H7 in a variety of raw and ready-to-eat food products.

The primers amplified a single product from each target bacterium. More recently, Kim *et al*., (2007) developed a novel mPCR assay for the simultaneous screening of five

foodborne pathogenic bacteria including *Salmonella*. Specific primers for mPCR amplification of the Shiga-like toxin gene (Stx2), femA (cytoplasmicprotein), toxR (transmembrane DNA binding protein), iap (invasive associative protein), and invA genes were designed to allow simultaneous detection of E. *coli* O157:H7, S. *aureus*, *Vibrio parahaemolyticus*, L. *monocytogenes, and Salmonella spp.*, respectively. Furthermore, the detection of all five food borne pathogenic bacteria could be completed in less than 24 h. similar approaches have been described by others utilizing various primer sets for a variety of pathogens (Li and Mustapha, 2004).

c. Reverse transcriptase PCR (RT-PCR)

In RT-PCR, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is then amplified using conventional, multiplex, or real-time PCR. For example, Rijpens *et al*., (2002) targeted the house keeping *rpoD* gene of *Salmonella.* Overall, the assay could not detect viable *Salmonella* in heat or ethanol killed *Salmonella* cells. However, conventional RT-PCR techniques are labor intensive since the amplicon can be visualized only after the amplification ends, requires the rapid extraction of RNA due to its short half-life, suffers from an increased cross-contamination risk of the samples thus requiring DNase treatments, and the target genes must demonstrate abundant transcript expression, expression throughout the growth cycle, and negligible or no transcriptional regulation (Deisingh and Thompson, 2004).

Due to these difficulties, the development of RT-PCR applications focusing on the detection of food-borne pathogens, including *Salmonella* in foods and environmental samples has been limited. D'Souza *et al*., (2009) developed a RT-PCR for the rapid detection of *Salmonella* using *invA* primers.

d. Nucleic acid hybridization

Probes directed to specific gene regions of the *Salmonella* genome provide a powerful tool for use in DNA hybridization assays. Such methods of detection have proven to be more sensitive than agarose gel electrophoresis and more specific than culture or immunological based assays (Manzano *et al*., 1998). Maciorowski *et al*., (1998) was able to detect PCR products from *S.* Typhimurium inoculated animal feeds by hybridization with biotin and fluorescently labeled probes.

Commercial hybridization assays for the detection of *Salmonella* include the Gene Quence *Salmonella* assay (Neogen) utilizing probes previously evaluated by D'Aoust *et al*., (1995). This test employs Salmonella-Specific DNA probes, which are directly labeled with horseradish peroxidase. A colorimetric endpoint is then used for the detection of *Salmonella* spp. in food samples following broth culture enrichment with results available within 24 h.

e. Phage based detection methods

Virulent phages with a broad host range within the *Salmonella* genus are ideally suited for detection purposes since they are unable to integrate into the host genome, with the successful infection always resulting in the death of their host (Hagens and Loessner, 2007).

Since the first report of the use of phage for detection different strategies have been described for the detection of *Salmonella*. Generally, the majority of methods described involve measuring the activity of a reporter gene (generally, the luciferase *lux* genes from *Vibrio fisherii*), cloned into a vector carried by a phage, and expressed only after infection (Thouand *et al*., 2008).

2.1.6. Treatment

Early treatment is essential for septicemic salmonellosis, but there is controversy regarding the use of antimicrobial agents for intestinal salmonellosis. Oral antibiotics

may be ineffective and may deleteriously alter the intestinal microflora, thereby interfering with competitive antagonism and prolonging shedding of the organism. There is also concern that antibiotic-resistant strains of salmonellae selected by oral antibiotics may subsequently infect people. By suppressing antibiotic-sensitive components of the normal flora, antibiotics may also promote transfer of antibiotic resistance from resistant strains of *E coli* to *Salmonella*. Use of chemotherapeutic antibiotics for growth stimulation has been banned in many countries for this reason (Gruenberg, 2014).

Broad-spectrum antibiotics administered systemically are indicated for treatment of septicemia. Initial antimicrobial therapy should be based on knowledge of the drug resistance pattern of the organisms previously found in the area. Nosocomial infections may involve highly drug-resistant organisms. Trimethoprim-sulfonamide combinations may be effective. Alternatives are ampicillin, fluoroquinolones, or third-generation cephalosporins. Resistance to ampicillin, trimethoprim, sulfonamide, tetracyclines, and aminoglycosides is generally plasmid mediated and transfers readily between different bacteria. Resistance to quinolones is mutational, but random mutations may be selected by antibiotic use and may be transferred by bacteriophages. Treatment should be continued daily for up to 6 days (Gruenberg, 2014)

If oral medication is chosen, it should be given in drinking water and not mixed into solid feed, because affected animals are thirsty due to dehydration and their appetite is generally poor. Fluid therapy to correct acid-base imbalance and dehydration may be necessary. Calves, adult cattle, and horses need large quantities of fluids. Antibiotics such as ampicillin or cephalosporins lead to lysis of the bacteria with release of endotoxin, and NSAIDs or flunixinmeglumine may be used to reduce the effects of endotoxemia (Gruenberg, 2014).

The intestinal form is difficult to treat effectively in all species. Although clinical cure may be achieved, bacteriologic cure is difficult, either because the organisms become established in the biliary system and are intermittently shed into the intestinal lumen, or because the animals are reinfected from the environment at a time when their normal gut flora, which is inhibitory to colonization by pathogens, is depleted by antibiotic therapy. A concern with antimicrobial therapy is that it may increase the risk of creating carrier animals; in people and other animal species, antimicrobial therapy prolongs the period after clinical recovery during which the pathogen can be retrieved from the GI tract (Gruenberg, 2014).

2.1.7. Prevention and Control

Carrier animals and contaminated feedstuffs and environment are major problems. Drain swabs or milk filters may be cultured to monitor the *Salmonellae* status of a herd. The principles of control include prevention of introduction and limitation of spread within a herd (Gruenberg, 2014).

Prevention of Introduction- Every effort must be made to prevent introduction of a carrier; ideally, animals should be purchased directly only from farms known to be free of the disease and should be isolated for ≥1 wk while their health status is monitored. Ensuring that feed supplies are free of salmonellae depends on the integrity of the source. Some countries also test for contamination of and regulate importation and home production of feedstuffs and feed components (Gruenberg, 2014).

Limitation of Spread within a Herd-In an outbreak of salmonellosis, the following procedures should be implemented: 1) Carrier animals should be identified and either culled or isolated and treated vigorously. Treated animals must be rechecked several

times before there can be confidence they are not carriers. 2) The prophylactic use of antibiotics in feed or water supplies may be considered (the hazards are mentioned earlier). 3) Movement of animals around the farm should be restricted to limit infection to the smallest group. Random mixing of animals should be avoided. 4) Feed and water supplies must be protected from fecal contamination. 5) Contaminated buildings must be vigorously cleaned and disinfected. 6) Contaminated material must be disposed of carefully. 7) All persons should be aware of the hazards of working with infected animals and the importance of personal hygiene. A strict farm management program should be introduced. 8) Use of a vaccine should be considered, particularly in an outbreak involving pregnant cattle, pigs, or laying poultry. Commercial killed bacterins or autogenous bacterins may be used. Live attenuated vaccines show considerable promise, but few are available commercially. 9) Stresses should be minimized (Gruenberg, 2014).

Salmonella Vaccines-Salmonellae are facultative intracellular bacteria, and a live vaccine is therefore expected to be necessary for optimal immune protection against disease; however, there is some evidence that inactivated bacterins can induce a lower level of protection. In several studies, live attenuated Salmonella vaccines in pigs, cattle, and chickens stimulated a strong cell-mediated immune response and protected animals against both systemic disease and intestinal colonization. A live attenuated S. Choleraesuis vaccine licensed for use in swine appears to effectively reduce colonization of tissues and protect pigs from disease after challenge with virulent organisms and under field conditions. This vaccine also protected calves against experimental challenge with S. dublin and serogroup C1 salmonellae after intranasal or SC administration. A live S. Gallinarum vaccine has been shown to be effective not only against S. Gallinarum (fowl typhoid) but also in significantly reducing the infection of laying hens challenged with S. enteritidis (Gruenberg, 2014).

2.2. Antimicrobials and Antimicrobial Resistance

Antimicrobials used in the therapy of infectious diseases are the drugs that either kill or suppress microorganisms such as bacteria, viruses and parasites. Antibiotics are the subgroup of antimicrobials that act only against bacteria. The actions of antibiotics are (i) inhibition of cell wall synthesis, (ii) inhibition of protein synthesis, (iii) inhibition of DNA/RNA precursor (folate) synthesis, (iv) inhibition of DNA/RNA synthesis, and (v) disruption of membrane proteins (Walsh, 2003).

The selective pressure refers to the impact of antimicrobial use on a microbial population, in which resistant organisms gain a survival advantage over those susceptible ones. According to Clinical Laboratory Standards Institute (CLSI), resistance designates the isolates that are not inhibited by "usually achievable systemic concentration of the agent with normal dosage schedules and/or fall in the range where specific antimicrobial resistance mechanisms are likely (e.g. beta-lactamases) and clinical efficacy has not been reliable in treatment studies" (CLSI, 2006).

A constant increase in the numbers of resistant strains has been observed since the 1950s. This is based on the distinctly higher selective pressure as imposed by the use of antimicrobial agents for various purposes in human and veterinary medicine, aquaculture and horticulture during the last 60 years. According to the bulletin of WHO in 2002, the mortality rate in outbreaks involving resistant strains of *Salmonella* spp. was found to be 3.4%, whereas it was only 0.2% in those sensitive strains (Smith and Coast, 2002).

Another concerning aspect of antimicrobial resistance is the multidrug resistance of pathogens, which makes the selection of antimicrobials more difficult in the clinical treatment of the disease. The most common *Salmonella* serotype having multidrug

resistance is S. Typhimurium definitive phage type DT104. It acquired multiple drug resistance, with an isolate from the United Kingdom found to display a phenotype of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT) so called penta-resistance. The zoonotic nature of DT104 may have provided the environment for the acquisition of the ACSSuT resistance phenotype since this resistance includes four of the five most common drug classes used in veterinary medicine (Mulwey et al., 2006). Later, it was seen that multidrug resistance phenotype is emerging in other *Salmonella* serotypes too. In the recent studies, serotypes such as *S.* Typhi, *S.* Paratyphi, *S.* Infantis, *S.* Uganda, *S.* Agona, and *S.* Newport, *S.* Hadar, *S.* Heidelberg are exhibited multidrug resistance in addition to *S.* Typhimurium (Zhao *et al*., 2007).

The resistance mechanisms can be classified as (i) destruction or modification of the antimicrobial agent, (ii) pumping the antimicrobial agent out from the cell by efflux pumps, (iii) replacement or modification of the antibiotic target, and (iv) reduction in cell membrane permeability. Microorganisms are developing resistance mechanisms by accumulating mutations in the gene locations of target proteins or acquiring mobile genetic elements carrying resistance genes (Walsh, 2003).

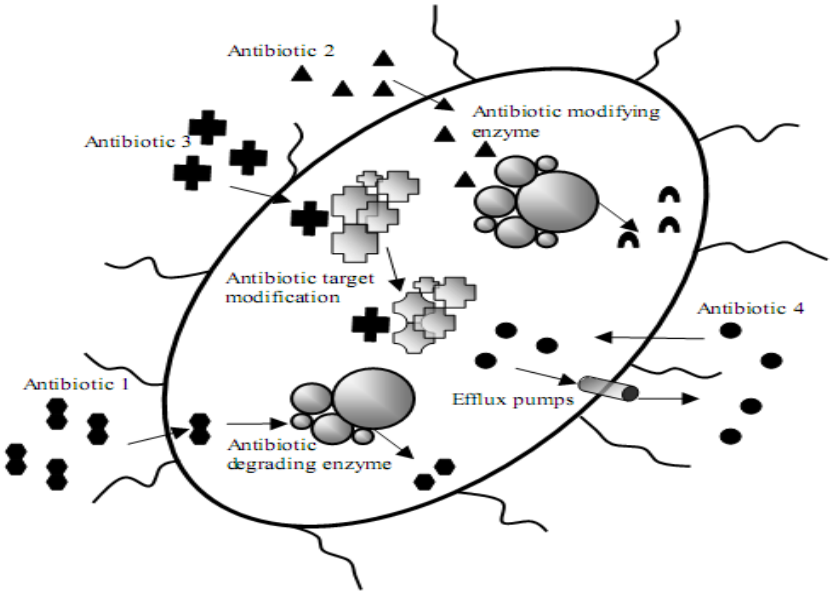

Fig.4. Schematic representation of the mechanisms of antimicrobial resistance (modified from http://www.chembio.uoguelph.ca/merrill/research/ enzyme_mechanisms. html).

There are several antimicrobial drug classes, namely β-lactams, quinolones/fluoroquinoles, aminoglycosides, phenicols, tetracyclines, sulfonamides and trimethoprim.

β-lactams are one of the critically important antibiotics in both human and veterinary medicine. Penicillins are also used for growth promoters of animals. β-lactams are bacteriocidal by blocking the trans-peptidations peptidoglycan layer of bacterial cell wall where they bind to penicillin binding-proteins. There are three major groups of β-lactams: penicillins, cephalosporins, and carbapenems. Possibly because of the widespread clinical use of penicillins, resistance to drugs such as ampicillin and methicillin has become common. In response to this problem, second class of β-lactams, the cephalosporins, was developed. Penicillins have a five-member thiazolidine ring fused to the β-lactam ring. Cephalosporins are the enzymatically converted form of penicillins by a ring expandase enzyme forming a six-member ring (a dihydrothiazine ring) fused to the β-lactam ring (Alcaine *et al*., 2007).

These changes provide cephalosporins with a broader range of activity and greater stability in the presence of β-lactamases. Both penicillins and cephalosporins are fungal secondary metabolites produced by Penicillium chrysogenum (Walsh, 2003).

There are four generations of cephalosporins, and each progressive generation is effective against a broader range of organisms. Although Salmonella isolates may appear susceptible to first and second-generation of cephalosporins in vitro, the CLSI cautions that this antimicrobial drug class may not be clinically effective against *Salmonella* soon (Alcaine *et al*., 2007).

The latest discovered group of β-lactams is carbapenems, which differs from penicillins and cephalosporins by lacking sulphur in five-membered ring fused to the four-member

β-lactam ring. These β-lactams are sometimes paired with β-lactamase inhibitors. Carbapenems have a much broader range of activity against both Gram-negative and Gram-positive bacteria than do other β-lactams and are more stable against β-lactamases. Nevertheless, *Salmonella* isolates that possesses resistance to carbapenems such as imipenem already have been reported (Singh *et al*., 2007).

β-lactam resistance mechanism in *Salmonella* is mostly mediated by the production of the enzymes β-lactamase. These enzymes work by hydrolysing the β-lactam ring structure, yielding beta-amino acids with no antimicrobial activity. The genes encoding for β-lactamases produced by *Salmonella* are typically carried on plasmids, although most of these genes are chromosomally encoded in other bacterial species. β-lactamases are classified by two schemes called Ambler classes A-D and Bush classes 1-4. Ambler's classification scheme is based on the primary structure and amino acid sequence identity of β-lactamases. According to Ambler's classification scheme there are class A, B, C, and D beta-lactamases. In general, class A β-lactamases are the most commonly reported class of β-lactamases in *Salmonella*. They are plasmid encoded and provide a range of resistance against penicillins, early generation cephalosporins, and carbapenems. There are several different gene families encoding for enzymes in this class, and blaTEM-1 is the most prevalent among *Salmonella* isolates. Other Class A β-lactamase gene such as blaPSE-1 also has been found in a number of *Salmonella* isolates and chromosomally encoded (Li *et al*., 2007).

The emergence of cefotaximases (CTX-M), which are class A β-lactamases conferring resistance pirmarily to ceftiofur, is an important trend to watch. Variants of blaCTX-M have been identified in isolates of *Salmonella* serotypes (Livermore *et al*., 2007).

Class B β-lactamases are metallo-β-lactamases which are not commonly found in *Salmonella*.

Class C β-lactamases are typically encoded by chromosomal ampC genes and provide resistance against cephalosporins and ceftiofur. *Salmonella* has no chromosomal ampC

gene; instead, these genes are harbored in plasmids. Currently researches are primarily focused on blaCMY-2, which has been associated with resistance primarily to cephoxitin. The spread of blaCMY-2 is a public health concern because the presence of this gene appears to mediate resistance or at least reduced susceptibility to ceftriaxone, another extended spectrum cephalosporin that is the drug of choice for the treatment of *Salmonella* infections in children (Livermore *et al*., 2007).

Class D β-lactamases appear to be rare among Salmonella isolates. This class of enzymes provides resistance to β-lactams closely related to oxacillin and methicillin. The chromosomally encoded gene blaoxa-1 (=blaoxa-30) was found in a *S.* Paratyphi, *S.* Muenchen and *S.* Typhimurium. This group is resistant to inhibitors such as clavulanic acid (Alcaine *et al*., 2007).

Quinolones and fluoroquinolones are synthetic bacteriocidal drugs. In 1962, nalidixic acid became the first quinolone approved for medical use. Several generations of quinolones have been developed, with each new generation having improved action against bacterial infections. The early generation quinolones target DNA gyrase, and the late generation quinolones both DNA gyrase and topo-isomerase IV. The mode of action for quinolones is quite complex and not completely understood. Although quinolones target topoisomerases, they do not actually bind to the topoisomerase but to the double stranded DNA in the topoisomerase complex (Alcaine *et al*., 2007).

There are documented cases of *Salmonella* isolates with resistance to nalidixic acid and low-level resistance to fluoroquinolones and high-level resistance to quinolones to be emerging. Quinolone resistance of Salmonella isolates has been linked to two mechanisms, target gene mutations and active efflux. The first mechanism is mediated by target mutations in the quinolone resistance determining region (QRDR) of gyrA and gyrB, the two genes that encode the subunits of DNA gyrase, and in the parC subunit of topoisomerase IV. The most frequently amino acid substitutions observed in gyrA are Ser-83 (to Phe, Tyr, or Ala) or Asp-87 (to Gly, Asn, or Tyr) and inparC is Thr-57 (to Ser) (Cloeckaert and Chaslus-Dancla, 2001).

The second mechanism involves changes in the expression of the AcrAB-TolC efflux system, mostly due to mutations in the genes encoding regulators of this system (e.g., marRAB) that results in over expression and consequently decreased quinolone sensitivity. No single mutation confers high-level resistance to fluoroquinolones but resistance results from the accumulation of multiple mutations. The facts that Salmonella isolates must acquire multiple unlinked mutations and that some of those mutations reduce fitness, particularly those involved in the regulation of the efflux pump, may explain why this kind of resistance is so infrequent (Alcaine *et al*., 2007).

Quinolone resistance also has been linked to the expression of the plasmid-mediated qnr gene. This gene codes for a protein that appears to bind to DNA gyrase and protect it from quinolone inhibition. Research conducted on plasmids harboring qnr revealed that this gene could be transferred from other bacterial species to *Salmonella* via conjugation. Although documented cases of plasmid-mediated quinolone resistance in Salmonella isolates are rare, a recent study indicated that the spread of such plasmids to Salmonella isolates has also occurred (Kehrenberg *et al*., 2007).

The appearance of plasmid-mediated quinolone resistance in *Salmonella* isolates is a very important emerging public health concern. Plasmids harboring qnr also can harbor other resistance genes, suggesting that the treatment of infections with *Salmonella* strains containing this plasmid may be increasingly difficult. In a recent study, reduced susceptibility ciprofloxacin was conferred by a variant of the gene encoding aminoglycoside acetyltransferase AAC (6')-Ib. Even if the gene was detected among other Enterobacteriaceae, it has to the best of our knowledge that this gene has not been identified yet in Salmonella isolates (Kehrenberg *et al*., 2007).

Aminoglycosides were first discovered in 1944 from Streptomyces griseus and since have been widely used. Other aminoglycosides are kanamycin, neomycin, amikacin, and gentamicin. They are hydrophilic sugars with multiple amino groups and target 16S rRNA on the 30S ribosome from the A site of aminoacyl-tRNA binding which leads to

codon misreading and translation inhibition. Most aminoglycosides are bactericidal (destructive), with the exception of spectinomycin, which has a bacteriostatic (growth inhibiting) mode of action (Walsh, 2003; Alcaine *et al*., 2007).

Resistance to aminoglycosides in Salmonella is mainly associated with the modification of aminoglycoside molecules by enzymes. These enzymes fall into three groups that are named according to the types of reactions they catalyse (Alcaine *et a*l., 2007): Aminoglycoside acetyltransferases are enzymes that primarily acetylate aminoglycoside-amino groups. Genes encoding these enzymes are typically designated aac and these genes have been found as part of *Salmonella* genomic islands, integrons and plasmids. Aminoglycoside acetyl-transferases provide resistance to gentamicin, tobramycin, and kanamycin. Aminoglycoside phosphotransferases are enzymes that catalyze ATP-dependent phosphorylation of specific aminoglycoside hydroxyl groups. Most genes encoding these enzymes are designated as aph provide resistance to kanamycin and neomycin. The genes aph(3')-Ib and aph(6)-Id are commonly referred in the literature as strA and strB, respectively and provide resistance to streptomycin. Nucleotidyl-transferases also target the hydroxyl groups. Genes encoding nucleotidyl-transferases are usually designated aad (for aminoglycoside adenyltransferases), although some are also designated as ant (for aminoglycoside nucleotidyltransferase). The aadA gene [or ant (3')] provides streptomycin resistance in Salmonella isolates. The aadB gene [or ant (2')-Ia] contributes resistance to gentamicin and tobramycin. Both aadA and aadB have been found as integron-borne gene cassettes (Sørum and L'Abée-Lund, 2002).

Phenicols include chloramphenicol and florfenicol. Chloramphenicol was once the drug of choice for the treatment of typhoid fever. Production of chloramphenicols by Streptomyces venezuelae was discovered in 1947. Chloramphenicol works by binding to the peptidyltransferases centre of the 50S ribosomal unit, thus preventing formation of peptide bonds. Chloramphenicol's broad range activity against Gram-positive and Gram-negative bacteria and its ability to cross the blood-brain barrier make it a powerful choice for the treatment of systemic infections. Its toxicity, which can lead to bone marrow damage and aplastic anemia, and widespread resistance have generally limited

chloramphenicol use to occasions where the risk of the infection, such as bacterial meningitis, is greater than the risk of adverse effects from the drug. Chloramphenicol is still widely used in developing countries because of its low cost (Walsh, 2003; Alcaine *et al.*, 2007).

Chloramphenicol resistance in *Salmonella* isolates is conferred through two mechanisms: (i) the enzymatic inactivation of the antibiotic via chloramphenicol O-acetyltransferase (CAT) and (ii) the removal of the antibiotic via an efflux pump. The genes encoding for CAT are plasmid-borne and commonly found in S. Typhi isolates. CAT genes, such as cat1 and cat2, have also been found in non-typhoidal *Salmonella* serotypes. Chloramphenicol efflux pumps in Salmonella isolates have been reported to be encoded by two closely related genes, cmlA and floR. The floRgene appears to be widespread in *Salmonella* isolates, whereas cmlA is less widely distributed. The highly mobile floR gene has been found in Salmonella genomic islands and in many different plasmids. It appears to be associated with multidrug resistance (Walsh, 2003; Alcaine *et al.*, 2007).

Tetracyclines were discovered in the 1940s. The first tetracycline, chlorotetracycline, was isolated from Streptomyces aerofaciens. Tetracyclines were popular because of their minimal adverse effects and broad-spectrum activity. They were effective against most bacteria, including chlamydias and mycoplasm, and even some protozoa. Tetracyclines act by preventing the binding of tRNA to the A site of the 30S ribosomal subunit, thus inhibiting protein synthesis. Unfortunately, the rise of resistant bacteria has severely limited the use of tetracycline (Walsh, 2003; Alcaine *et al*., 2007).

Tetracycline resistance of *Salmonella* isolates is attributed to production of an energy-dependent efflux pump, which removes this antimicrobial drug from the bacterial cell. Other mechanism of resistance, such as modification of the ribosomal target and enzymatic inactivation of tetracycline, have been attributed to other bacterial species but have yet to be reported in Salmonella isolates. Deletion or inactivation of marRAB

operon also has been linked to the reduced susceptibility to tetracycline (Walsh, 2003; Alcaine *et al*., 2007).

There are at least 32 different genes that confer resistance to tetracycline and oxytetracycline. Of these, tet(A), tet(B), tet(C), tet(D), tet(G), and tet(H) have been found in Salmonella isolates. The most commonly reported one of these genes is tet (A). It has been found in Salmonella genomic island 1, on integrons, and on transferable plasmids. The tet(A) gene has been detected in isolates of Salmonella serotypes. Like tet(A), tet(B) has also been located on transferable plasmids. These genes appear to be easily transferred and widespread among Salmonella isolates. They also tend to be found in isolates that display multidrug resistance, making them an important marker in identifying potentially serious Salmonella infections. Tet (G) is linked to *Salmonella* Genomic Island 1 (SGI1) (Walsh, 2003; Alcaine *et al*., 2007).

Sulfonamides and Trimethoprim prescribed separately and has been used in combination for the treatment of bacterial infection since the late 1960s. These compounds are bacteriostatic antimicrobial drugs that act by competitively inhibiting enzymes involved in the synthesis of tetrahydrofolic acid. Sulfonamides inhibit dihydropteroate synthetase (DHPS) and trimethoprim by inhibiting dihydrofolatereductase (DHFR). The combination of a sulfonamide and trimethoprim has been a popular form of treatment for decades, and although resistance among Salmonella isolates has emerged, this resistance does not appear to be common (Walsh, 2003; Alcaine *et al*., 2007).

Sulfonamide resistance in *Salmonella* isolates has been attributed to the presence of an extra *sul* gene, which expressed an insensitive form of DHPS. Three main sul genes have been identified: sul1, sul2, sul3. The sul1 gene has been found in a wide range of Salmonella serotypes. This gene is often associated with class I integrons that contain other resistance genes. These integron-borne gene cassettes have been found on transferable plasmids and as part of Salmonella genomic island variants. Although sometimes found in Salmonella isolates also harbouring sul1, sul2 appears to be associated with plasmids, but not with class I integrons. Isolates of Salmonella serotypes

Agona, Enteritidis, Typhimurium have been reported to carry sul2. The sul3 gene has been identified only recently in Salmonella, and it has been associated with plasmids and class I integrons, suggesting that there may be further dissemination of this gene within Salmonella populations (Guerra *et al*., 2004a). Deletion or inactivation of the marRAB also has been linked to reduced sulfonamide susceptibility.

Similar to sulfonamide resistance, trimethoprim resistance is attributed to the expression of DHFR that does not bind trimethoprim. There are mini variants of the dhfr an ddfr genes that encode this resistance, such as *dhfr1, dfrA1, and dhfr12.*These genes have been found as part of integron borne gene cassettes also associated with*sul1* and *sul3,* on transferable plasmids carrying other resistance genes, and *Salmonella* genomic islands.

In recent years, *Salmonella* isolates obtained from humans and from food-producing animals have exhibited resistance to an increasing variety of antimicrobials. A common serotype that is also often multi-resistant is Typhimurium. Recent studies have shown that, in some cases, over 90% of isolates obtained from food-producing animals are multi-resistant (Farrington *et al.,* 2001).

Among isolates obtained from humans, multi-resistance is not as common, but recent data show that 26% of all isolates, and 50% of serotype Typhimurium, are now resistant to at least one antimicrobial (NARMS, 2000). A common resistance among animal isolates is to tetracyclines, an antibiotic class used routinely in animal agriculture as a growth promoter. Resistance to several other antibiotics has been commonly found, including sulfamethoxazole, streptomycin, and ampicillin. Also of concern is decreasing susceptibility to two antimicrobials important in the treatment of human disease, ciprofloxacin and ceftriaxone. Although resistance to these two remains low (less than 2% of isolates from humans), it has increased significantly during the last several years (FDA/USDA/CDC, 2001).

Among both animal and human isolates, resistance to ampicillin, chloramphenicol, tetracycline, streptomycin, and sulfonamide is common and is most often associated with

phage type *DT104*, which has been responsible for numerous disease outbreaks (Poppe *et al.*, 1998). This phage type encodes its resistance factors as a chromosomal element, with physical linkage of all five resistance genes (Briggs and Fratamico, 1999).

Three of the resistance elements (ampicillin, streptomycin, and sulfonamide) are encoded on integrons, genetic elements capable of acquiring and exchanging resistance factors. A second common resistance pattern is to ampicillin, kanamycin, tetracycline, streptomycin, and sulfonamide, frequently associated with the phage type *DT193*. Unlike *DT104*, this phage type carries its resistance factors on one or more conjugative plasmids, and so can transfer its resistance factors to other *Salmonella* strains or bacterial species (Gebreyes *et al.*, 2000).

3. MATERIALS AND METHODS

3.1. Study Area

A cross sectional study was conducted between November 20013 to April 2014 DebreZeit town located at 9 ^{0}N and 40 ^{0}E. It is 47 km South East of Addis Ababa, thecapital of Ethiopia. The altitude is about 1850m above sea level. It experiences bimodal patterns of rainfall with the main rainy season extending from June to September with an average rainfall of about 800 mm. The mean annual minimum and maximum temperatures are 12.3 ^{0} C and 27.7 ^{0}C, respectively with an overall average of 18.7 ^{0} C. The mean relative humidity is 61.3% (CSA, 2011).

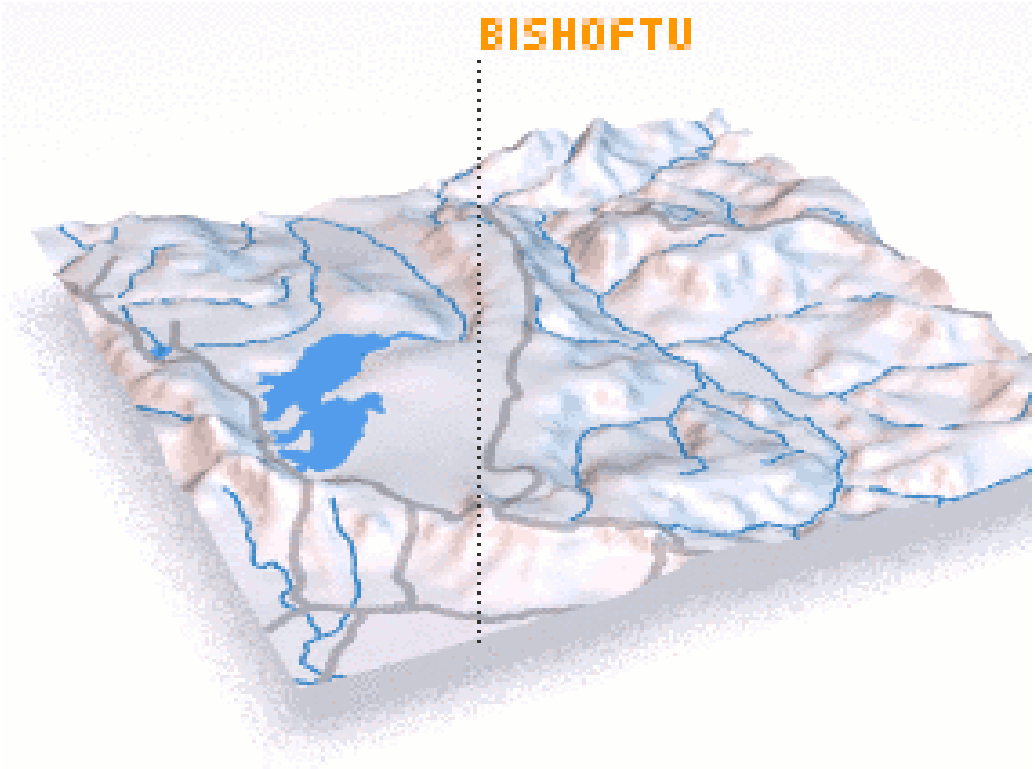

Fig 5. Map of Bishoftu Source:wikipedia

3.2. Sample Size Determination and Study Design

The sample size was determined by using $n = [1.96^2 P\text{exp}\ (1-P\text{exp})/\ d^2]$ (Thrusfield, 2007); Pexp = 10.78 (Addis 2011); d (absolute precision) = 0.05

Based on the above formula the calculated sample size was 148

Eight farms were selected from the list that has been provided from the city's municipality based on the type of the farms and a total of 148 apparently healthy dairy cattle were purposively selected and from each dairy farm samples were taken using simple random sampling technique.

3.3. Sample Collection

Milk samples were collected as described by Quinn *et al*., (1994). Ten ml of milk was collected in sterile tubes after dropping out the first three streams of milk. The fecal specimens of cows were also collected in a clean sterile air tight stool cup (universal bottle) directly from the rectum. Then the samples were held in an ice box and transported to the Microbiology laboratory of Addis Ababa University, College of Veterinary Medicine and Agriculture, Debre-Zeit for processing.

3.4. Bacteriological Examination of Milk and Fecal Samples

In this study to diminish the risk of obtaining false negative results, a non-selective pre-enrichment of the fecal and milk samples, a combination of two selective enrichments and plating on two selective media were performed.

Pre-enrichment of test samples in non selective medium: Pre-enrichment of the fecal and milk samples was achieved by inoculating 1ml of milk and 1gm of fecal samples in 9ml of buffered peptone water (BPW) each and incubating the samples 37^{o}c for 24 hrs (ISO 6579: 2002; Quinn, 2004**;** Spencer, 2001; FDA, 2001; Wray and Wray, 2001; OIE, 2010).

Selective enrichment of test samples: After the pre-enrichment a portion of the sample (0.1ml) was taken from milk sample and inoculated in Rappaport Vassilidis broth and another portion (1ml) was taken and transferred to Selenite F broth and incubated at 41.5^{o}c and 37^{o}c for 24 hrs respectively the fecal was incubated in the same way) (ISO 6579: 2002; Quinn, 2004; FDA, 2001; Wray and Wray, 2001; OIE, 2010)..

Subcultivation: For subcultivation the enriched samples were then taken and inoculated on selective media which was achieved by striking a loop full of sample from each enrichment media and by using a half plating technique on one dish; one half for culture on RV and another for culture on Selenite on both Xylose Lysine Deoxycholate agar and on Brilliant Green agar and incubating the media at 37^{o}c for 48 hrs (ISO 6579: 2002; Quinn, 2004; FDA, 2001; Wray and Wray, 2001; OIE, 2010).

Characteristic Salmonella colonies, having a slightly transparent zone of reddish color and a black center from XLD and white with pink background from BGA, were sub-cultured on nutrient agar and incubated at 37^{o}c for 24hrs to commence with biochemical tests (ISO 6579: 2002; Quinn, 2004; FDA, 2001; Wray and Wray, 2001; OIE, 2010).

Biochemical tests for conformation

All suspected non-lactose fermenting *Salmonella* colonies were picked from the nutrient agar and inoculated into the following biochemical tubes for identification: triple sugar iron (TSI) agar, lysine decarboxylate broth, urea broth, tryptone water, MR-VP broth and incubated for 24 or 48 hours at 37 ^{o} C. Colonies producing an alkaline slant with acid (yellow color) butt on TSI with hydrogen sulphide production, positive for lysine (purple color), negative for urea hydrolysis (red color), negative for tryptophan utilization (indole test) (yellow-brown ring), negative for Voges-Proskauer, and positive for citrate utilization were considered to be *Salmonella*-positive (Appendix e) (ISO 6579: 2002; Quinn, 2004**;** Spencer, 2001; FDA, 2001; Wray and Wray, 2001; OIE, 2010).

3.5. Antimicrobial Susceptibility Tests

The antimicrobial susceptibility test of the isolates was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) method using Kibry-Bauer disk diffusion test on Muller-Hinton agar (Oxoid CM0337 Basingstoke, England).

The isolates were tested for the following antibiotics; amoxaciline (10 µg), streptomycin (10 µg), kanamycin (30 µg), gentamycin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), trimethoprim (2.5 µg), cefoxitin (30 µg), cotrimoxazole (Thrimethoprimsulfmethoxazole) (25 µg), nalidixic acid (30 µg).

3.6. Data Management

The data collected during the study periods was entered into MS Excel software. The statistical analysis used includes comparison of proportions and Chi-square test, which was applied to test if statistically significant association existed between susceptibility of milk and fecal samples to antimicrobials. For all the analysis of prevalence $p<0.05$ was taken as statistically significant.

4. RESULT

From the total of 148 dairy cows tested 12.8% (19/148) were positive for salmonella, either from milk or feces. Of these cows 15.8% (3/19) were positive from milk and 84.2% (16/19) were positive from fecal samples.

Table 2. Salmonella positive cultures

Sample type	Number of samples positive (%)
Milk	3(15.8%)
Feces	16(84.2%)
Total	19(100%)

All 19 isolates were subjected to panel of eleven antimicrobials, the antimicrobial susceptibility pattern of the isolates indicated that 89.5%, 100%, 42.1%, 63.3%, 15.8%, 10.5%, 31.6%,42.1%, 52.6% resistant to amoxacyline, tetracycline and gentamycin, kanamycin, trimetoprim, streptomycin, cotrimoxazol, chloramphenicol, nalidixic acid and cefoxitin respectively no resistance has been found for ciprofloxacin.

Table 3. Antibiotic resistance pattern of salmonella isolates from milk and fecal samples

Antibiotics Tested	Resistant(%)	Intermediate(%)	Susceptible(%)
Amoxacyline	89.5%(17)	-	10.5%(2)
Tetracycline	100%(19)	-	-
Kanamycine	42.1%(8)	-	57.9%(11)
Trimetoprim	63.2%(12)	-	36.8%(7)
Streptomycin	15.8%(3)	21%(4)	63.2%(12)
Ciprofloxacin	-	10.5%(2)	89.5%(11)
Cotrimoxazol (Selphamethoxazole-thrimethoprim)	10.5%(2)	15.8%(3)	73.7%(14)
Chloramphenicol	31.6%(6)	26.3%(5)	42.1%(8)
Nalidexic acid	42.1%(8)	-	57.9%(11)
Gentamycin	100%(19)	-	-
Cefoxitin	52.6%(10)	-	47.4%(9)

Salmonella isolated from milk sample showed 100% resistance to amoxacilin, trimetoprim, tetracycline, nalidexic acid, gentamycin, cefoxitin and kanamycin 33.3 % to chloramphenicol while they were 100% susceptible to streptomycine, ciprofloxacine, cotrimoxazol. Fecal isolates of cows showed 100% susceptible ciprofloxacine, 12.5% cotrimoxazol and 100% resistance to tetracycin and gentamycin 87.5% to amoxicillin, 31.3% to chloramphenical nalidexic acid and kanamycin 56.3% to trimetoprim, 18.8 to

streptomycin and 43.8 % to cefoxitin. However no statistical significance $p>0.05$ has been seen between milk and fecal samples.

Resistivity of drugs	Sample taken			
	Milk (n= 3)	Fecal (n= 16)		
	Positive (%)	Positive (%)	X^2	P-value
Amoxicillin	3 (100.0)	14 (87.5)	0.4191	0.7018
Chloramphenicol	1 (33.3)	5 (31.3)	3.7076	0.1566
Trimetoprim	3 (100.0)	9 (56.3)	2.0781	0.2270
Tetracycline	3 (100.0)	16 (100.0)	-	-
Streptomycin	0 (0.0)	3 (18.8)	2.0781	0.3538
Ciprofloxacin	-	-	-	-
Nalidexic acid	3 (100.0)	5 (31.3)	4.8984	0.0577
Gentamycin	3 (100.0)	16 (100.0)	-	-
Cefoxitin	3 (100.0)	7 (43.8)	3.2063	0.1238
Cotrimoxazol	0 (0.0)	2 (12.5)	1.0933	0.5789

Table 4. Relation of drug sensitivity between milk and fecal samples

Kanamycin	3 (100.0)	5 (31.3)	4.8984	0.0577

Totally 8 Multiple Drug Resistance (MDR) pattern were also observed. The highest MDR was for three antibiotics with the combination Amc, Te, Cn being more frequent. In general MDR to three, seven and eight antibiotics dominate the resistance patterns (31.6%, and 21.1% each).

Table 5. Multidrug Resistance profile of salmonella species isolated from milk and fecal samples

No. antibiotics resistant	Antimicrobial resistace pattern(no. of isolates)	No of isolates(%)
3	Amc,Te,Cn(4)	6(31.6)
	Te,S,Cn(2)	
4	Amc,Te,C,Cn(1)	1(5.3)
5	Amc,Te,W,S,Cn(2)	2(10.5)
6	Amc,Te,W,S,Cn,Fox(1)	2(10.5)
	Amc,Te,Sxt,Cn,Fox(1)	
7	Amc,Te,K,W,Na,Cn,Fox(4)	4(21.1%)
8	Amc,Te,K,W,C,Na,Cn,Fox(4)	4(21.1)

The prevalence of salmonella in each farm was found to be 0, 11.1, 15, 7.7, 31.25, 25, 20 and 11.1 percent in farm 1,2,3,3,4,5,6,7,8 respectively and the isolates have shown multidrug resistance and Amc,Te,Cn being seen redundantly this can be due to the use of Tetracycline as IM for most of illnesses and Gentamycin as an intra-mammary infusion for mastitic treatment in most farms and clinics in the town.

Table 6. Multidrug Resistance pattern of salmonella in each farm

No. farms	Antimicrobial resistance pattern (No. of isolates)	No of isolates (%)
F2	Amc,Te,K,W,C,Na,Cn,Fox(2)	2(10.5)
F3	Amc,Te,W,C,Cn(1)	3(15.8)
	Amc,Te,W,S,Cn(1)	
	Amc,Te,W,Sxt,Cn,Fox(1)	
F4	Te,S,Cn(1)	2(10.5)
	Amc,Te,K,W,Na,Cn,Fox(1)	
F5	Amc,Te,Cn(1)	5(26.4)
	Amc,Te,K,W,Na,Cn,Fox(2)	
	Amc,Te,K,W,C,Na,Cn,Fox(2)	
F6	Amc,Te,Cn(1)	2(10.5)
	Amc,Te,K,W,Na,Cn,Fox(1)	
F7	Amc,Te,Cn(1)	3(15.8)
	Te,S,Cn(1)	
	Amc,Te,W,S,Cn,Fox(1)	
F8	Amc,Te,Cn(1)	2(10.5)
	Amc,Te,C,Cn(1)	

4. DISCUSSION

In this cross sectional study the overall prevalence of antimicrobial resistance of *Salmonella* from148 dairy cattle was found to be 12.8% which is comfortably in line with a study of Addis *et al*., (2011) at 10.76% from milk and fecal samples and other studies, even though most of the reports are from slaughtered cattle from abattoirs and ready to eat food items (Zewdu, 2009; Alemayehu, 2003; Ejeta, 2004) and milk products, it was found to be lower than the study by Tadesse and Dabassa, (2012) at 20% from raw milk. The difference in prevalence might be the use of enrichment media in this study.

Of the total positive cows for *Salmonella* 15.8% (3/19) were positive from milk and 84.2% (16/19) were positive from fecal samples. Comparison between feces and milk in this study has shown no significant difference. And this is in line with the study by Addis *et al*., (2011).

In the other study by Addis *et al.* (2011) from 195 dairy cows tested 28.6% were positive from milk samples. Akoachere *et al.*, (2009) in Cameroon reported a high prevalence (27%) of *Salmonella* among cattle. Steele *et al*.(1997) detected *Salmonella* in only 0.17% of bulk tank samples from Ontario, Canada, Murinda *et al.* (2002) found *Salmonella spp*. in 2.24% of milk samples from the bulk tanks of 30 Tennessee farms and Elahe *et al*.(2012) found 4% in raw milk in Iran. This might be due to the difference in the living condition, like housing conditions, feeding habits, types of feed given for the cattle.

In this study *Salmonella spp*. isolated from milk sample showed 100% resistance to amoxacilin, trimetoprim, tetracycline, nalidexic acid, gentamycin, cefoxitin and kanamycin, 33.3 % to chloramphenicol while they were 100% susceptible to streptomycine, ciprofloxacine, cotrimoxazol.

However salmonella resistant to tetracycline 100% and gentmicyne 100% were found higher in present study as compared to the findings of Tadesse and Dabassa, (2012) tetracycline 35% and gentmicyne 25%. This might be due to high antimicrobial use in dairy farms and individual cows bacterial contaminants carried by milk and milk products often shows high level of antimicrobial resistance (Sandgren *et al.*,2008).

Multi drug resistance for at least three or more antimicrobials was found to be much higher than other studies conducted in Ethiopia (Addis *et al*., 2011; Dabassa and Bacha, 2012; Tadesse and Debasa, 2012). This difference may be due to the increasing rate of inappropriate utilization of antibiotics in the dairy farms which favors selection pressure that increased the advantage of maintaining resistance genes in bacteria (Mathew, 2007; Mcgree, 1998).

Ciprofloxacin showed good antimicrobial activityin milk and in fecal isolates, in this study and no resistance was detected. This is also comparable with the result reported by (Addis *et al*., 2011; Tadesse and Debasa, 2012; Akinyemia *et al*., 2005; Hawkey, 2008; Sandgren *et al*., 2008). The effectiveness of such drugs like ciprofloxacin may be because they are not widely and used in animals in countries like Ethiopia and other African countries.

Cotrimoxazole (trimethoprim-sulfametoxazole) has also showed a good antimicrobial activity against most isolates. This result is lower than the reports by Rotimi *et al*., (2008) from Kuwait and United Arab Emirates who reported a resistance rate of 26.1% and 8.9%, respectively. Even though cotrimoxazole has been widely available the reason of its effectiveness until this times need investigations.

In this study multidrug resistance patterns in the sampled farms was observed and most of the isolates were resistant to Tetracycline and Gentamycin which is in line with the study by Dabassa and Bacha, (2012). The reason for wide spread resistance development could be due to the frequent use of the drugs in veterinary services as a broad spectrum antibiotics.

5. CONCLUSION and RECOMENDATIONS

Examination of the prevalence and multidrug resistance pattern from milk and fecal samples of apparently healthy animals in dairy farms gives a very good idea about the development and spread of drug resistant salmonella in dairy farms of debrezeit.

Most of the studies done in Ethiopia show salmonella prevalence in different milk and meat products and not on apparently healthy dairy cattle and this study gives an insight in that the apparently healthy cattle serve as a carriers to multidrug resistant salmonella and may transmit infection to susceptible animals.

The study also shows that drug resistance is becoming higher by the day which might be due to inappropriate use of drugs in farms.

Based on the above conclusion the following recommendations have been forwarded:-

- More studies specially on apparently healthy animals should be performed as they might be carries and may hider successful prevention and control mechanisms
- Antibiotic use especially in farms should be monitored as animal products are consumed raw most of the time in our country and this could lead to acquiring drug resistant salmonella infections
- Use of broad spectrum antibiotics for treatment should be kept in line to minimize the risk of development of resistance by micro-organisms
- Farm owners should be made aware of the risks of use of an monitored drugs, there management system and sanitization for them not to be impacted by infectious diseases.

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7. APPENDICES

Appendix A Sample plating recording sheet

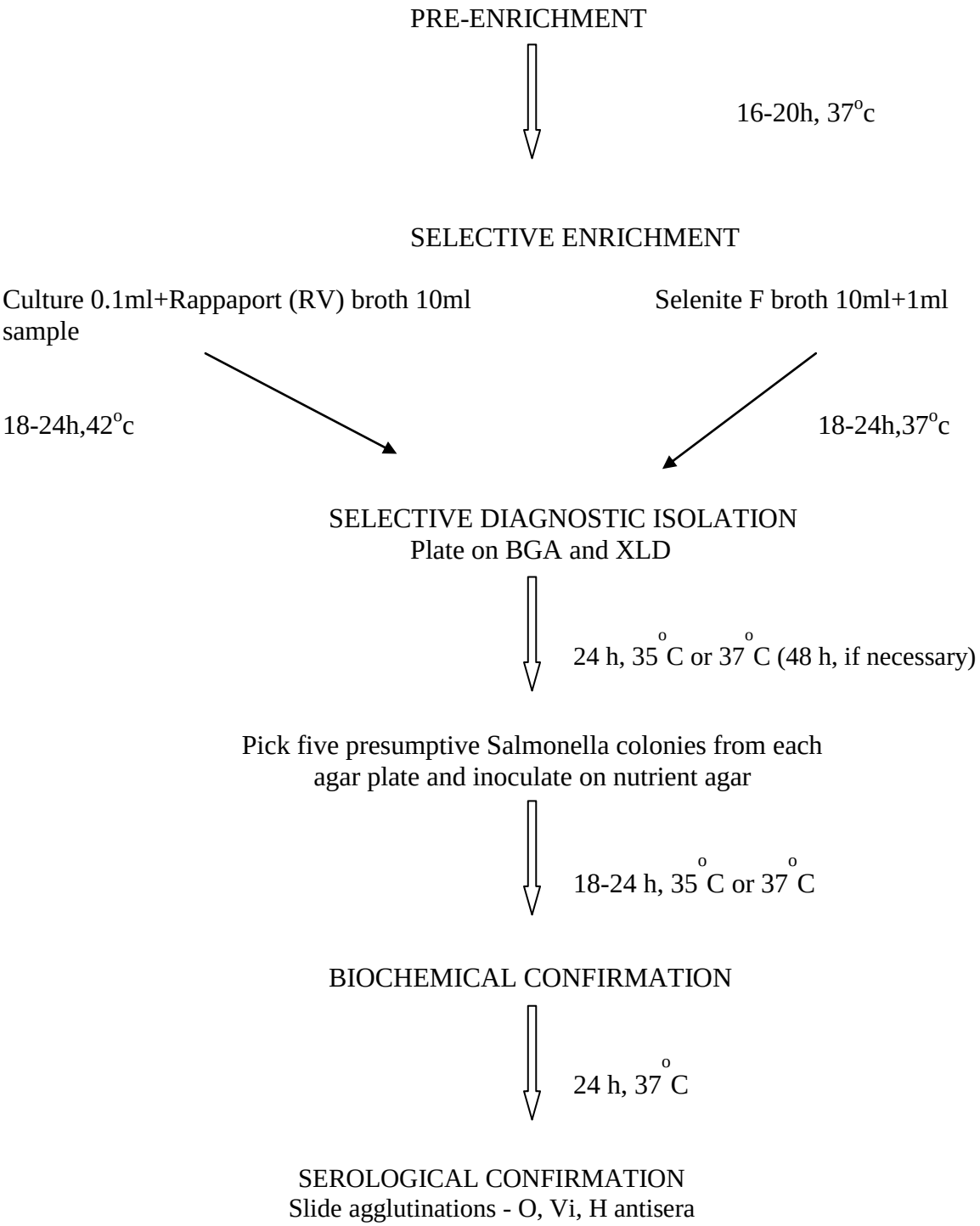
Sample_____ Place _____Enumerator _____

date	Sample no.	BGA		XLD		Sample+/-	comment

Appendix B Biochemical test record sheet

Date	Sample and colony Id	TSI				Urea	L-lysine	VP	Indole	Salmonella +/-	comment
		butt	slant	gas	H_2S						

Appendix C BSI/ISO Salmonella Isolation Procedure



Appendix D Composition and method of preparation of media

1. Buffered Peptone Water (BPW) – SIFIN, Berlin, Germany

Typical formula (g/l):

- Peptone from casein…… ……10.0
- Sodium chloride ……5.0
- Di-sodium hydrogen phosphate……..3.5
- Potassium dihydrogen phosphate……1.5

Direction: Dissolve 20 g in 1 liter distilled water and sterilize by autoclaving at 121^{o}C for 15 minutes.

2. Selenit-F broth of 500 g (SIFIN, Berlin, Germany)

Typical formula (g/l):

- Peptone from casein……..5.0
- L(-)cystine…….0.01
- Lactose……..4.0
- Phosphate buffer …….10.0
- Sodium hydrogen selenite …….4.0

Direction: Suspend 23 g in 1 litre of de-mineralized water at room temperature. If necessary, warm shortly (max. 60 ^{o}C) filter-sterilize if storage is planned; dispense into suitable containers. Don't autoclave. pH :7.0 ±0.2 at 25 ^{o}C

3. Rappaport – Vassiliadis (RV) enrichment broth of 500 g (Oxoid, England)

Typical formula (g/l):

- Soya peptone …….5.0 g
- Sodium chloride……..8.0 g
- Potassium dihydrogen phosphate …..1.6 g
- Magnesium chloride …….40.0 g
- Malachite green…….0.04 g

Directions: Weigh 30 g (the equivalent weight of dehydrated medium per liter) and add to 1 liter of distilled water. Heat gently until completely dissolved. Dispense 10 ml volumes into screw-capped bottles or tubes and sterilize by autoclaving at 115 ^{o}C for 15 minutes. This medium is very hygroscopic and must be protected from moisture.

4. Brilliant Green Phenol Red Agar acc. to Edel and Kampelmache (BPLS Agar, mod.) of 500 g. (Sifin, Berlin, Germany).

Typical formula (g/l):

- Peptone…… 10.0
- Meat extract……5.0

- Yeast extract……3.0
- Lactose……..10.0
- Saccharose…….10.0
- Disodium hydrogen phosphate……1.0
- Sodium dihydrogen phosphate…….0.6
- Brilliant green……0.005
- Phenol red……..0.09
- Agar…….10.0

pH 7.0 ± 0.2

Directions: Suspend 49.7 g in 1 litre of distilled water; carefully bring to the boil with frequent agitation to dissolve completely. Don't autoclave! Mix well and pour into petridishes.

5. XLD – Agar acc. ISO 6579 (Xylose-lysine-desoxycholate agar) of 500 g (Sifin, Berlin, Germany).

Typical formula (g/l):

- Yeast extract…….. 3.0
- L-Lysine hydrochloride……..5.0
- Xylose…….3.75

- Lactose……....7.5

- Sucrose……7.5

- Sodium deoxycholate……..1.0

- Sodium chloride……..5.0

- Sodium thiosulphate……6.8

- Iron (III) ammonium citrate……0.8

- Phenol red……...0.08

- Agar……..16.5

pH: 7.4 ± 0.2

Directions: Suspend 57 g in 1 liter of distilled water; carefully bring to the boil with frequent agitation to dissolve completely. Don't autoclave! Mix well and pour into Petri dishes. Storage: Dry, tightly closed, at 10 – 25 ^{o}C.

6. Nutrient agar of 500 g (Oxoid, England).

Typical formula (g/l):

- Lab-Lemco powder……..1.0

- Yeast extract ……2.0

- Peptone…….5.0

- Sodium chloride……....5.0

- Agar……15.0

pH: 7.4 ± 0.2

Directions: Suspend 28 g in 1 litre of distilled water. Bring to the boil to dissolve completely.

Sterilize by autoclaving at 121^{o}C for 15 minutes.

7. Triple Sugar Iron Agar (Difco, Detriot, USA)

Approximate formula per liter:

- Beef extract…….3.0 g
- Yeast extract……3.0 g
- Pancreatic digest of casein……...15.0g
- Proteose peptone N0. 3……..5.0g
- Dextrose…….1.0g
- Lactose……..10.0g
- Sucrose…….10.0g
- Ferrous sulfate……0.2g
- Sodium chloride…….5.0g
- Sodium thiosulfate……0.3g
- Agar……..12.0g
- Phenol red……0.024g

Directions: Suspend 65 g of powder in 1 litre of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Dispense into tubes and autoclave at 121 ^{o}C for 15 minutes. Cool in a slanted position so that deep butts are formed.

8. Lysine Decarboxylase Broth of 500 g (Difco, Detroit, USA).

Formula per litre:

- Bacto peptone……5 g
- Bacto yeast extract……3 g
- Bacto dextrose……...1 g
- L-lysine…….5 g
- Bacto Brom Cresol Purple… ……...0.02 g

Directions: Suspend 14 grams in 1 litre distilled water or deionized water and boil to dissolve completely. Sterilize at 121 – 124 ^{o}C for 15 minutes. Final pH: 6.8 ± 0.2 at 25 ^{o}C.

9. Urea Broth (Merck, Germany)

Typical formula (g/l):

- Yeast extract ……0.1
- Potassium dihydrogen phosphate…….9.1
- Disodium hydrogen phosphate…….9.5
- Urea…….20.0

- Phenol red ……..…0.01

Preparation: Dissolve 38.5 g/l, if necessary heat up to a temperature of 60 ^{o}C. Sterilize by filtration or dispense aliquots of approximately 3 ml into test tubes and sterilize for 5 minutes in a current of steam under mild conditions. Don't autoclave!. pH = 6.8.±0.1. The broth is clear and yellow-orange. If filter sterilization or heat sterilization is not possible, the medium must be inoculated as soon as it has been prepared.

10. MR- VP Medium (Buffered glucose broth) (glucose phosphate broth) (Himedia laboratories Pvt.Ltd, Mumbai, india):

Compositions (g/litres):

Buffered Peptone…… 7

Dextrose…….. 5

Dipotassium phosphate…… 5

pH=6.9 ± 0.2 at 25 ^{O}C.

Preparations; suspend 17 g in 1000ml distilled water. Heat if necessary to dissolve the medium completely, distribute in test tubes in 10 ml amount and sterilize by autoclaving at 15 Ibs pressure at (121 ^{o}C) for 15 minutes.

11. Reagents for VP test

1-Naphihol, ethanolic solution.

Composition; 1-Naphihol 6gm; ethanol, 95% (volume fraction) 100ml

Preparation; dissolve the 1-naphihol in the ethanol.

Potassium hydroxide solution

Compositon; potassium hydroxide 40gm; water; 100ml

Preparation; dissolve the potassium hydroxide in the water

12. Tryptose soya broth (Oxoid LTD, BASINGSTOKE, hampshire, England):

Compositions (g/litres):

Pancreatic digest casein…… 17

Enzymatic digest of soya bean……..... 3

Sodium chloride……. 5

Di-potassiun hydrogen phosphate…… 2.5

Glucose…… 2.5

pH= 7.3 ± 0.2 at 25 ^{O}C.

Preparations; dissolve 30 g in 1 liter of water (purifies, as required) an distribute in to final containers. Sterilize by autoclaving at 121^{o}C for 15 minutes.

13. Reagent required for indole test

Kovac's reagent(UNI_CHEM) readymade.

Composition:

4-dimethyl-aminobenzaldehyde…….5gm

Ethanol alcohol……75ml

Hydrochloric acid…… 25ml

Appendix E Biochemical test indicators

Biochemical test indicators	Test reactions	Percent
TSI acid	+	100
TSI gas	+	91.9
TSI lactose	-	99.2
TSI sucrose	-	99.5
TSI H_2S	+	91.6
Urea splitting	-	99
Lysine decarboxylation	+	94.6
β-galactosidase rxn	-	98.4
Voges-Proskauer rxn	-	100
Indol reaction	-	98.9

Appendix F Zone diameter interpretive standard chart for Enterobacteriacea

Antimicrobial agents and symbols	Disc contents(µg)	Zone diameter, nearest whole mm		
		Resistance	Intermediate	Susceptible
Trimetoprim(W)	2.5	≤ 13	14-16	≥ 17
Nalidexic acid(NA)	30	≤ 15	16-17	≥ 18
Gentamycin(CN)	10	≤ 12	13-14	≥ 15
Kanamycin(K)	30	≤ 13	14-17	≥ 18
Streptomycin(S)	10	≤ 11	12-14	≥ 15
Trimethoprim-Sulfamethoxazole(SXT)	1.25/23.75	≤ 10	11-15	≥16
Amoxacillin(AML)	20	≤ 13	14-16	≥ 17
Tetracyclin(TE)	30	≤ 11	12-14	≥ 15
Chloramphenicol(C)	30	≤12	13-17	≥ 18
Cefoxitine (FOX)	30	≤ 14	15-17	≥ 18
Ciprofloxacin(CIP)	5	≤ 14	15-17	≥ 21

Source: http://www.eucast.org/expert_rules/ (2012)