ISOLATION, IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF *LISTERIA MONOCYTOGENES* FROM RAW BOVINE MILK IN DEBRE-BIRHAN TOWN, NORTH SHOA, ETHIOPIA.

MSc. Thesis

BY:

YESHIBELAY GIRMA

JANUARY, 2015

JIMMA, ETHIOPIA

JIMMA UNIVERSITY
COLLEGE OF AGRICULTURE AND VETERINARY MEDICINE
SCHOOL OF VETERINARY MEDICINE

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A Thesis submitted to the School of Graduate Studies of Jimma University, College of Agriculture and Veterinary Medicine School of Veterinary Medicine in partial fulfillment of the requirements of the degree of Master of Science in Veterinary Public Health.

JANUARY, 2015
JIMMA, ETHIOPIA

DEDICATION

I would like to dedicate this paper to my families

STATEMENT OF AUTHOR

First I declare that this thesis is my own research efforts and all sources of material used for this thesis have been duly and appropriately acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc. Degree in Veterinary Public Health at Jimma University, College of Agriculture and Veterinary Medicine, School of Veterinary Medicines and is deposited at the University/ College library to be made available to borrow under the rules of the library. I somberly declare that this thesis is not submitted to any other institute for the award of any academic degree, diploma or certificate.

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Name: Yeshibelay Girma, Signature: _____

Place: Jimma University, Jimma

Date of submsion: _____

BIOGRAPHICAL SKETCH

Yeshibelay was born in September, 18th 1984 G.C from his father Girma Woldemedihn and mother Yidenekal Tiruneh in North Shoa Zone, Berehet, Metitehbila town, Ethiopia. He attended his primary elementary and junior school at Metitehbila primary elementary and junior school (1992 to 1998 G.C), secondary school at Ginager secondary school (1999 to 2000 G.C) and accomplished his preparatory education at Debre birhan Hail mariam Mammo Secondary and preparatory school starting from 2001 to 2002 G.C. After completion of his preparatory education, he joined Haramaya University, Faculty of Veterinary Medicine in 2003/04 G.C and attended Veterinary Medicine and he received his undergraduate Doctor of Veterinary Medicine (DVM) in 2008 G.C. Following graduation from Haramaya University he worked in North Shoa Zone, Berehet woreda in Agricultural office as Animal health team leader and Veterinarian of the woreda for four years. He joined Jimma University for MSc. Program in Veterinary Public Health (VPH) from March 2013 G.C to date.

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ABBREVIATIONS

AFNOR	Association Francaise de Normalization
AOAC	Association of Official Analytical Chemists
AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
BCM	Biosynth Chromogenic Medium
BLEB	Buffered *Listeria* Enrichment Broth
CAMP	Christie Atkins Munch Peterson test
CDC	Center for Disease Control and Prevention
CFSAN	Center for Food Safety and Applied Nutrition
Cfu	Colony forming unit
CSF	Cerebrospinal Fluid
FAO	Food and Agricultural Organization of the United Nations
FDA	Food and Drug Administration of the United States
FSIS	Food Safety and Inspection Service
IDF	International Diary Federation
Inl	Internalin
ILSI	International Life Sciences Institute
ISO	International Organization for Standardization
LEB	*Listeria* Enrichment Broth
LLO	Listeriolysin O
LPM	Lithium Chloride-phenylethanol-moxacalatam medium
MFA	Modified Fraser Agar
MOX	Modified Oxford Agar
NCCLS	National Committee for Clinical Laboratory Standards
OXA	Oxford Agar
PALCAM	Polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin Mannitol
RTE	Ready to eat
Spp	Species

TSYEB	Tryptone Soy Yeast ExtractBroth
TSYEA	Tryptone Soya Yeast Extrac Agar
USDA	United States Department of Agriculture
UVM	University of Vermont *Listeria* enrichment medium
L	*Listeria*
χ^2	Chi-square

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ABSTRACT

Listeriosis is a disease of humans and animals, in which it is one of the important emerging bacterial zoonotic diseases worldwide. Among the different species of *Listeria, Listeria monocytogenes* is the most common causes of Listeriosis in humans and animals, as a result of food and environmental contamination with low incidence but high case fatality rate. The present study was undertaken to isolate *Listeria* species in raw bovine milk. A cross-sectional study was conducted from January to September 2015 to determine the presence of *Listeria monocytogenes* and other *Listeria* species from raw milk samples originated from dairy producers and vendors and determining antimicrobial resistance profile of *Listeria monocytogene*. A total of 407 raw milk samples of which, 384 from dairy producers were collected by using simple random sampling technique and 23 from vendors were also collected. *Listeria* species isolations were performed according to the standard bacteriological techniques by using *Listeria* enrichment broth, Modified Fraser broth and Oxford Agar medium as well as comfirmatory tests: carbohydrate utilization (rhamnose, xylose, mannitol); blood agar (hemolysis) and Christie Atkins Munch Peterson (CAMP) test. The antimicrobial resistance profile of *Listeria monocytogenes* was also assessed by using the standard disk diffusion method (Kirby Bauer techniques) and it was tested against 9 antimicrobial drugs (Cephalothin 30µg, Chloramphenicol 30µg, Kanamycin 30µg, Nalidixic acid 30µg, Streptomycin 10µg, Tetracycline 30µg, Vancomycin 30µg, Gentamicin 10µg and Ampcillin 10µg). Overall isolated *Listeria* species were *Listeria monocytogenes* 36 (8.84%), *Listeria innocua* 28 (6.88%), *Listeria seeligeri* 14 (3.4%), *Listeria grayi* 3 (0.74%), *Listeria welshimeri* 2 (0.49%) and *Listeria murrayi* 2 (0.49%). Antimicrobial susceptibility test was conducted on 36 isolated *Listeria monocytogenes. Listeria monocytogenes* were found to be resistant to two or more antimicrobial. The presence of *Listeria monocytogenes* in raw milk and drug resistant isolates of the bacteria is an indication of public health hazards to the consumers, particularly to the high risk groups. Hence awareness creation on milk safety and implementations of regulations about the use of antimicrobials in humans and animals should be strongly practiced.

Keywords: Antimicrobials, Debre-birhan, Detection, Isolation, *Listeria monocytogene*, milk, Susceptibility.

1. INTRODUCTIONS

The genus *Listeria* is a group of closely related Gram-positive, facultative anaerobic, non-spore forming, rod shaped, and motile bacteria. Genus *Listeria* comprises ten species, i.e., *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L.murrayi, L. marthii, L. fleischmannii,* and *L. weihenstephanensis,* (Meloni, 2014). *Listeria monocytogenes* is the principal pathogen in humans and animals. *Listeria ivanovii* is a pathogen of animals but is occasionally implicated in human disease. The other *Listeria* species are generally considered as non-virulent (Liu, 2006).

Listeria monocytogenes, the causative agent of listeriosis is ubiquitous in the environment and has been recognized as animal pathogen since the 1920s but in the past two decades, it has been implicated in several outbreaks of food-borne illness in humans (Pintado *et al.,* 2005; Yucel *et al.,* 2005). Contamination of silage leads to infection of farm animals resulting in possible human infection by way of the food chain (Ramaswamy *et al.,* 2007; Sleator *et al.,* 2009). Food-borne transmission constitutes the main acquisition route of listeriosis (Churchill *et al.,* 2006).

In a wide variety of host animals *Listeria* infection has been confirmed in more than 40 species of domestic and wild animals including birds. The most susceptible domestic species are sheep, goats and cattle. Listeriosis manifests itself clinically in ruminants as encephalitis, neonatal mortality (abortion) and septicaemia. The most common clinical form in cattle is encephalitis, in general, small numbers being affected (8-10% of the herd) with the animals surviving from 4-14 days (Quinn *et al.*, 2002).

In humans, food-borne *L. monocytogenes* causes large outbreaks of Listeriosis, with a mortality rate of 9% to 44% (Clark *et al.,* 2010). Certain sections of the population are predisposed to the development of listeriosis due to presence of existing chronic illness, suppression of the immune system, pregnancy, or extreme youth or age (under 1 year or over 60 years) (Ramaswamy *et al.*, 2007). This presents a significant public health problem because in such population, listeriosis is fatal in up to 30% of cases (Aurora *et al.,* 2008)

which may increase up to 75% in high risk groups such as pregnant women, neonates and immune-compromised persons (Jalali and Abedi, 2008).

Listeria monocytogenes the leading cause of death among bacterial pathogens acquired primarily through the consumption of contaminated foods (Jemmi and Stephan, 2006). Transmission is generally through eating contaminated food, particularly dairy products made from unpasteurized milk and ready-to-eat meat and fish products (EFSA, 2009). The incidence of *L. monocytogenes* in soft and semi-soft cheese varied from 0.50% to 46.00% (Manfreda *et al.,* 2005; Pintado *et al.,* 2005; Colak *et al.,* 2010).

Raw milk can be contaminated from the environment or by direct excretion into the milk; therefore, consumption of raw milk is associated with increased risk factors. *L. monocytogenes* has been largely studied in the past decades because of its importance as a food-borne human pathogen (Ryser and Marth, 2004; Saran, 2013). Ongoing efforts are needed to further reduce the incidence of listeriosis, due to the manifestation of its high mortality rate (ILSI Research Foundation-Risk Science Institute, 2005; www.esb.ucp.pt/isopol 2010).

Listeria monocytogene detection and identification in foods traditionally involve culture methods based on selective pre-enrichment, enrichment and plating. This is followed by the characterization of *Listeria* species using colony morphology, sugar fermentation and haemolytic properties (Gasanov *et al.,* 2005; Paoli *et al.,* 2005). In the last years there has been a notable development of new culture media for the improved detection of *L. monocytogenes* in foods, and efficient methods based on antibodies or molecular techniques have also been developed (Gasanov *et al.,* 2005; Paoli *et al* ., 2005).

In developing countries most of the time, there have been few or no reports on *L. monocytogenes*. This is true because no one has given it attentions or awareness on the occurrence of *L. monocytogenes* in food (Molla *et al.*,2004). However, in Ethiopia a few studies were undertaken on the status of *L. monocytogenes* and other species. According to Muhammed et al., (2013) study in Jimma town, from a total of 200 food sample (milk, cottage cheese, ice cream and yoghurt) collected from November 2010 to March 2011, 13(6.5%) were positive for *Listeria* species. Prevalence of *Listeria* was higher 7(14%) in raw

milk samples followed by ice cream 3(6%), yoghurt 2(4%) and cottage cheese 1(2%). Studies by Abera (2007) in Addis Ababa from 240 food samples tested, 66(27%) were positive by conventional culture technique for *Listeria* spp., and from 59 viable isolates were raw meat 62.7%, raw milk 8.5%, cottage cheese 6.8%, and cream-cake samples 22.0%, respectively.

In Ethiopia, there is inadequate information regarding to the prevalence and antimicrobial susceptibility patterns of *Listeria* species (Garedew *et al.*, 2015) and there is no studies conducted in Debre-Birhan town on raw bovine milk from dairy producers. Therefore the present study was carried out to address the following objectives.

General objective:

To investigate the occurrence of *Listeria* in raw milk and to assess the presence of antimicrobial resistance profile.

Specific objectives:

- To isolate *L. monocytogenes* from raw bovine milk.
- To detect the presences of *Listeria* species other than *L. monocytogenes.*
- To determine the antimicrobial susceptibility pattern of *L. monocytogenes* isolates from raw milk.

2. LITERATURE REVIEW

2.1. Genus *Listeria*

2.1.1. Taxonomy and classification

The genus *Listeria* belongs to the Order Bacillales and family *Listeriaceae*. *Listeria* is a group of closely related Gram-positive, facultative anaerobic, non-spore forming, rod shaped, and motile bacteria. The genus *Listeria* comprises ten species, i.e., *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L. murrayi, L. marthii, L. fleischmannii, L. weihenstephanensis,* (Meloni, 2014) but *L. monocytogenes* is the principal pathogen in humans and animals. *L. ivanovii* is a pathogen of animals but is occasionally implicated in human disease. The other *Listeria* spps are generally considered as non-virulent (Liu, 2006).

2.1.2. Morphology

Members of the genus *Listeria* are non-spore-forming, facultative anaerobic and small Gram-positive rods (0.5–4 µm in diameter and 0.5–2 µm in length). Peritrichous flagella give them a typical tumbling motility, occurring at 20–25 ℃ (Meloni, 2015). The bacterial colonies are small, smooth, slightly flattened and milky white by reflected light (Salyers and Whitt, 2002).

2.1.3. Growth requirements

2.1.3.1. Effects of temperature

Listeria monocytogenes is a psychrophilic bacterium capable of growing at refrigeration temperature (-1.5 to 4°C). The optimal growth temperature is between 30^0C and 37^0C. But it can grow between 1^0C and 50^0C (Kasalica, 2011). *L. monocytogenes* can survive under adverse conditions although the organism is not heat-resistant and can be inactivated by pasteurization such as 72°C (161°F) for 15 seconds. Due to these characteristics of the organism it is very difficult to control *L. monocytogenes* for food safety (Kasalica, 2011).

2.1.3.2. Effects of water activity (a_w) and salt solution

Listeria monocytogenes is tolerant to high concentration of sodium chloride and capable of growth in 10% NaCl, it can stay alive for a year in 16% of NaCl. The optimum water activity

(a_w) for *Listerial* growth is ≥ 0.97, but *L. monocytogenes* being able to grow at water activity (a_w) less than 0.93 (Gandhi and Chikindas, 2007; Kasalica, 2011).

2.1.3.3. Effects of pH

Listeria monocytogenes and other species can grow best at the pH values that range from 4.5-9.6. The maximum growth pH of the bacterium depends on temperature of incubation, nutrient composition of growth substrate, water activity (a_w) and the presence of NaCl and other salts or inhibitors (Scott, 2003; Lado and Yousef 2007). Because of their decisive effect on the physiology of the bacterium the above-mentioned factors are known as growth conditions and survival limit (Todd, 2006; Lado and Yousef 2007) (Table1)

Table 1. Growth conditions and survival limits of *L. monocytogenes*

Parameter	Minimum	Maximum	Optimal	Survival without growth
Temperature (°C)	-1.5 to 3	45	30 to 37	-18
pH	4.2 to 4.3	9.4 to 9.5	7.0	3.3 to 4.2
Water activity	0.90 to 0.93	> 0.99	0.97	<0.90
Salt (%)	<0.5	12 to 16	-	≥20

Source: (Todd, 2006)

2.1.3.4. Growth and nutrition

Growth of *Listeria* spp. on semi-solid motility media is characterized by unusual umbrella-shaped growth in the surface. *Listeria* species grow on nutrient and blood agar but not on Mac-Conkey agar. Small transparent colonies with smooth borders appear on blood agar in 24 hours becoming grayish white and 0.5 to 2 mm in diameter in 48 hours (Quinn *et* al., 2002*)*. The bacterial colonies are small, smooth, slightly flattened and milky white by reflected light. Incubation of a 2 to 4 hour broth culture of *L. monocytogenes* at room temperature shows a tumbling motility, by hanging-drop method.

2.1.4. Biochemical characteristics

Listeria species are characterized by a wide range of biochemical characteristics most of which are used in the laboratory for the identification of the microorganism (Quinn *et al.*, 2002).

Table 2. Biochemical characteristics of *Listeria* species

Characteristics	*Listeria* species						
	L.mon	*L.ivanovii*	*L.seeligeri*	*L.innocua*	*L.welshimeri*	*L.grayi*	*L.murr*
Catalase test	+	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-	-
Indol test	-	-	-	-	-	-	-
Hydrolysis of aesculin	+	+	+	+	+	+	+
Hydrolysis of urea	-	-	-	-	-	-	-
Voges-Prosakuer	+	+	+	+	+	+	+
Fermentation of: mannitol	-	-	-	-	-	+	+
Xylose	-	+	+	-	+	-	-
Rhamnose	+	-	-	V	V	-	+

L. mon = Listeria monocytogenes, L. murr= Listeria murrayi; + =Positive, - = negative, V=variable; Source: (OIE Terrestrial Manual, 2014)

2.1.5. Typing of *Listeria*

Regulatory identification of *L. monocytogenes* does not require any specific sub typing of the isolates. However, sub-typing schemes can be useful in specific outbreaks, investigating, environmental tracking and public health surveillances (Graves *et al.*, 2010).

2.1.5.1. Sero-typing

Based on somatic (O) and flagellar (H) antigens, there are 13 serotypes of *L. monocytogenes* have been recognized. These are identified alphanumerically: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Meloni, 2015). Serotypes 1/2a, 1/2b and 1/2c are the most frequently isolated from food or the food production environment.

All serotypes are considered to be potentially pathogenic. However, only the three serovars, 1/2a, 1/2b and 4b are involved in the majority of animal and human listeriosis cases. Among the human isolates, 90% belongs to serovars 1/2a, 1/2b and 4b (Leclercq *et al.*, 2011). There are also three lineages; Lineage I (Highly pathogenic, with epidemic colony and responsible for most outbreaks 1/2b, 3b, 4b, 4d, 4e), Lineage II (medium pathogenic, sporadic cases 1/2a, 1/2c, 3c, 3a) and Lineage III (low and rarely cause human diseases 4a, 4c) (Arun, 2008). The lineage status of serotypes 4ab and 7 still remains unclear due to limited availability of such strains (OIE, 2014). Serotype 4b has caused the majority of human epidemics, while serotype 4a and 4c are the most common in animals. Sporadic cases of listeriosis are caused in animals by 4b, 1/2a and 1/2b (Irene *et al.*, 2003) (Table. 3).

Table 3: Serovars of *Listeria* species

Listeria species	Sero types
L. monocytogenes	1/2 a, 1/2 b, 1/2 c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, "7"
L. ivanovii	"5"
L. innocua	4ab, 6a, 6b
L. welshimeri	6a, 6b
L. seeligeri	1/2 b, 4c, 4d, 6b

Source: (FDA and CFSAN 2003).

2.1.5.2. Phage typing

Bacterio-phage typing is a technique with a very good discriminatory power and that can be used to subtype a large number of isolates. However the current available phage sets are unable to type a high proportion of strains (20-51% in case of International phage typing set). Because of stringent standardization requirements and the biological nature of the reagents,

this technique is performed at specialized National or International reference laboratories. Phage typing remains the most practical and convenient method for application in large and acute outbreaks (Graves *et al.*, 2010).

2.1.5.3. Genetic and molecular typing

DNA-based sub typing methods allow for differentiation of *L. monocytogenes* beyond the species and subspecies level. This sub typing method is improved not only the ability to detect and track animal and human listeriosis outbreaks but also provide tools to trace sources of *L. monocytogenes* contamination throughout the food chain (Irene *et al.*, 2003).

Genetic and molecular sub-typing include Pulsed-field gel-electrophoresis (PFGE), ribo-typing as well as PCR based formats such as randomly amplified fragment length of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) (Irene *et al.*, 2003). Pulsed-field gel-electrophoresis (PFGE) characterizes bacteria into subtypes by generating DNA banding patterns after restriction digestion of bacterial DNA with restriction enzymes. In PFGE sub typing method, restriction enzyme is used to cut the specific part of bacterial DNA into few large fragments while in ribo-typing restriction enzymes that are used in initial digestion of DNA are used to cut the DNA into many different fragments (Wiedmann and Evans, 2011).

2.1.6. Antimicrobials resistance

Due to the multiplicity of antibiotics, infections caused by drug-resistant bacteria did not represent a medical problem until the early 1980s. However, the selective pressure exerted by over prescription of drugs in clinical settings and their heavy use as growth promoters for farm animals has considerably accelerated evolution of bacteria towards resistance (Charpentier and Courvalin, 2002).

Listeria monocytogenes is intrinsically resistant to cephalosporins (cefazolin, ceftiofur, cefpirome), quinolones (nalidixic acid and early fluoroquinolone such as floxacine), fosfomycine and clindamycine. Acquired resistance has been rarely identified. Most of the isolates are susceptible to Penicillin G, amoxicilline, aminoglycosides (gentamycin), tetracyclines, phenicols, trimethoprim and sulfonamides, rifampin, glycopeptides

(vancomycin) (Granier *et al.*, 2011). At very low frequencies, resistance to tetracycline has been identified from various sources: beef meat, beef processing plants, pork cheeks, and sheep. Resistance to erythromycin was also identified in environmental and food samples. According to Granier *et al.*, (2011), report no remarkable evidences about penicillin resistance.

2.2. Epidemiology

Listeria species are found worldwide and everywhere, in animals, in foods, in humans, in soil, in the food processing environment, in contact surfaces and also in food containers (FDA and CFSAN, 2003). In the United States (US) the notification rate for listeriosis in 2010 was 0.27 cases per 100,000 populations. This was similar to the 2009 rate of 0.28 cases per 100,000 populations (CDC, 2012). In the European Union (EU) there were 0.32 confirmed cases of listeriosis per 100,000 populations in 2011 (ranging from 0.04–0.88 cases per 100,000 populations between countries). This was a 7.8% decrease in the number of cases from 2010. The fatality rates reported in EU in 2011 was 12.7% (EFSA, 2013). Of the several species of *Listeria*, *L. monocytogenes* is an important cause of wide spectrum of diseases in animals and humans. Species of *Listeria* have been isolated from at least 37 species of mammals (some literatures suggested about 42 wild and domestic mammals), 17 species of birds, and from others like flies, ticks, fish, crustaceans, and oysters (Todar, 2008).

Listeria monocytogenes is detected in different types of food and its prevalence rate differs from place to place, based on the hygiene, food content and environmental contamination rates. It was reported by several workers, in pasteurized milk and milk products, 2-30% (Molla *et al.*, 2004; Sarkar, 2006), in minced beef 7-36%, in meat products 0-52%, (Yang *et al.*, 2006), in vegetables, salads 1-12%, (Sagoo *et al.*, 2003),

The incidence of listeriosis in humans is very low, in the order of 3-8 cases per million inhabitants per year in the industrialized countries (Johan *et al.*, 2004). Most countries within the EU have an annual incidence between 2-10 reported cases per million populations per year (Valk *et al.*, 2005). Most cases of listeriosis are sporadic. Despite this, food borne outbreaks due to *L. monocytogenes* have been associated with cheese, raw (unpasteurised) milk, deli meats, salad, fish and smoked fish, ice cream and hotdogs (Montville and

Matthews, 2005; Swaminathan and Gerner-Smidt, 2007). Even though, the incidence of human listeriosis is low about (2-10/million) as indicated above, the over all fatality rate is 20 to 30% . The mortality rate can as high as 70% in untreated neurologic disease. The combination perinatal and neonatal mortality rates from 19% to 63% have been reported. The case fatality rate in infected newborns is approximately 50% (CFSPH, 2005).

For example, people with Human Immunodeficiency Virus (HIV) are almost 300 times more likely to get listeriosis than other healthy people. In addition, pregnant women are about 20 times more at risk than others and about 1/3 of listeriosis cases occur during pregnancy, late in 2^{nd} or in 3^{rd} trimester or 3 weeks of the newborn life. Moreover, about 370 unborn or newborn children are infected annually in the United States. Of these, 80 die and 56 suffer with lifelong complications (www.bfhd.wa.gov., 2003).

Due to *L. monocytogenes*, a number of major outbreaks of human listeriosis have been reported (Morrow *et al.*, 2004). The first confirmed food-borne outbreak of listeriosis occurred in 1981 in Nova Scotia, Canada, and involved 41 patients. Another outbreak that involved over 100 people was reported. Of these 34 occurred in pregnant women, among whom there were nine still births, 23 infants born infected and two live healthy births. There was also 30% mortality among 77 non pregnant adults (Todar, 2008).

The incriminated food at the origin of the invasive listeriosis outbreaks, which were reported in Europe, was found to be processed meat products (six outbreaks), cheese (five outbreaks), processed fish products (three outbreaks), butter (one outbreak) and undetermined (four outbreaks). The outbreaks of gastroenteritis were linked to the consumption of contaminated rice and corn salad respectively, while the Belgian outbreak of gastroenteritis and invasive listeriosis was linked to a contaminated ice cream cake whereas the origin of one outbreak of gastroenteritis remained undetermined (Valk *et al.*, 2005).

In Europe, between 1991 and 2002, a total of 19 outbreaks of invasive listeriosis were reported in nine different countries, with a total of 526 outbreak related cases while the number of reported outbreaks increased gradually over time, from seven outbreaks detected in the period 1992-1996 to eleven in 1997-2001. In addition, four outbreaks of acute *Listerial* gastroenteritis were reported: Of these, Italy had two outbreaks in 1993 (18 cases) and in 1997

(1566 cases); and one was in Denmark in 1996 (3 cases); and the fourth was in Belgium in 2001 (2 cases of acute gastroenteritis and one case of invasive Listeriosis) (Valk *et al.*, 2005). The mean incubation period of non-invasive gastroenteritis, which occurred in 1997, in Italy was reported to be 1-2 days, with diarrhea lasting from 1-3 days. The microorganism has gained recognition as food borne bacterium because of its association with epidemic gastroenteritis (Morrow *et al.*, 2004).

In Australia, there were also two reported outbreaks. In 1990 and 1991, six women gave still births and three ill people were reported, respectively. Laboratory tests confirmed the presence of *L. monocytogenes* (107 cells /gram) after checking samples of mussels that was eaten by the infected people. Similarly, in 1992, there were 270 reported cases of listeriosis in France that resulted in 63 deaths and 22 abortions, after eating pork tongue in jelly (FDA and CDC, 2003).

In developing countries like Africa and Latin America, most of the times, there have been few or no reports on *L. monocytogenes* (Molla *et al.*, 2004). This might be true because no one has given it due attention or were unaware of its occurrence. However, nowadays there are some reports on prevalence of *L. monocytogenes* in different samples. For example, in Nigeria, occurrence of *L. monocytogenes* in environmental samples and vegetables were studied and reported as 85% from cow dung, 91.6% from soil and 73.75% from vegetables (David and Odeyemi, 2007). In Ethiopia, there was also a work done, in 2004, which showed a prevalence rate of *Listeria* species about 31.6% and *L. monocytogenes* 5.1% in some foods, such as, meat, cheese, fish, pork, poultry, ice-cream (Molla *et al.*, 2004). Other reports studied by Gebretsadik *et al.* (2011) in Addis Ababa showed that the contamination levels of *Listeria* species to different food items such as cottage cheese and milks were ranging from 4% to 22% respectively. More recent study's under taken in Gondar town by Garedew *et al.* (2015), a total of 384 food samples examined, 96 (25%) were positive for *Listeria* species. According to this study the highest and the lowest prevalence of *Listeria* were found in raw meat 31(51.66%) and cottage cheese 5(12.5%) respectively.

Listeria monocytogenes is transmitted from man to man or from animal to man mostly through ingestion of the organism with contaminated food. It was reported that,

approximately 99% of human listerioisis appear to be food-borne. After entry following ingestion, the organism has a long incubation period, 1-90 days. In immuno-competent individual, listeriosis may occur mostly after being exposed to higher doses of the organisms. However, exposure of vulnerable individuals to even low doses may end up in acquiring listeriosis (Todar, 2008). The clinical course of infection usually begins about 20 hours after the ingestion of heavily contaminated food in case of gastroenteritis, whereas the incubation period for the invasive illness is around 20 to 30 days or much longer. Similar incubation period have been reported in animals for both gastroenteritis and invasive disease (Vazquez-Boland *et al.,* 2001)

The common risk factor for *Listeria* infection are food storing time, temperature, type of product, infective dose, immunity, and traditionally accepted consumption of raw foods. The following factors might contribute to the occurrence of high incidence of listeriosis in the future by increased proportion of susceptible people due to age, immuno-compromised diseases or treatment; increased use of cold storage to prolong the shelf life of foods; consuming of raw foods like raw milk or cheese from unpasteurized milk etc., especially which are known to harbor dangerous pathogens (Oliver *et al.,* 2005).

For example, *L. monocytogenes* has been reported several times from raw milk, in different countries; in USA, in 1987 (4 .2%), in 1992 (4.1%), in 1997 (4.6%); in Canada, in 1988 (1.3%, and 5.4%), and in 1998 (2.7%); in South Africa, in 1990 (5.2%); in Ireland, in 1992 (4.9%) and in England and Wales (5.1%). Surprisingly, the National Association of State Departments of Agriculture (NASDA) of US, presented that, the 2011 data shows that 30 states allowing to sale raw milk to the community at the company level (NASDA, 2011). Whereas the dairy farmers of Canada discussed historical overview of Canadian raw milk laws and made it clear that, sales of raw milk are illegal across Canada and can lead to fines of up to $250,000. Quebec is the only province that allows for raw milk cheese production. Such similar trend like the Canadian law has to be stated everywhere in order to protect the consumers, from such devastating bacterial pathogens (Chapman, 2006).

2.3. Public Health Importance

2.3.1. Listeriosis

Listeriosis is infectious disease of humans and animals, caused in 99% of cases by consumption of food contaminated by *L. monocytogenes*, and rarely from the environment (Todar, 2009). The majority of listeriosis occurs by consuming heavily contaminated food (McLauchlin *et al.,* 2004). Although the number of food borne outbreaks associated with *L. monocytogenes* is not high, the mortality rates are very high. According to the Center for Disease Control and Prevention, 1600 cases of listeriosis occur annually; 260 cases are fatal (CDC, 2011). The estimated annual number of cases of food borne salmonellosis, caused by *Salmonella*, is 14 million cases, and 400 cases are fatal (CDC, 2010).

In comparison to salmonellosis, the fatality rate of listeriosis is quite high. Also, sporadic cases appear to be more common than outbreak cases (Sauders and Wiedmann, 2007). The host's immune system, mainly via cell-mediated immunity (CMI), attacks the pathogen to prevent infection (Todar, 2011). However, if the host's immune system is compromised, some bacteria can survive the host's CMI and invade the intestinal mucosa and spread through intracellular mechanisms, causing serious infection (McLauchlin *et al.,* 2004). In particular *L. monocytogenes* can penetrate the blood-brain barrier and the placental barrier, causing severe infection of the brain and fetus, respectively (Doyle, 2001).

2.3.2. Food contaminations

2.3.2.1. Sources of contamination

Listeria monocytogenes is widely spread in nature easily enters the food and lead to contamination of the food. The bacteria was isolated in the soil; vegetation; water (sweet, salty and sewerage); raw and processed food (milk and dairy products); production facilities (ranges, floors, forklifts , washing tubs, working tables, knives, cutting equipment and machines, aprons, ripening premises, cold stores, open space, etc.) secretions of sick individuals (Todda and Notermansb, 2010).

Raw milk is one of the most common paths for transmission of *L. monocytogenes*, mainly due to sick animals on the farm. It is important to point out that healthy animals are often carriers

of *L. monocytogenes* and as such can be source of contamination of the environment, or milk. There is the opinion that the main source of contaminations of animals by *L. monocytogenes* is poor quality of prepared silage (Kasalica, 2011).

2.3.2.2. Contamination Pathways

Listeria is widely present in the environment, and there are many potential pathways by which food may be contaminated. Contaminated soil or water may introduce *Listeria* to produce in the field. Food producing animals may carry *Listeria*, often without symptoms, and be a source of contamination for milk and meat. Biofilms containing *Listeria* in food production and processing facilities may constitute a persistent, ongoing, sometimes sporadic source of bacteria (Srey *et al.*, 2013). Employees handling food may also spread *Listeria* and facilitate cross-contamination in production facilities and food preparation areas. Floor drains in poultry processing plants and other plants can be colonized by *L. monocytogenes* and the biofilms can make the drains difficult to clean (Berrang and Frank, 2012).

Listeria monocytogenes can produce extracellular polymers that aid in attaching to a variety of surfaces and protect the cells from cleaning and sanitizing agents. Once a biofilm is established, it can serve as an ongoing source of contamination. *L. monocytogenes* strains vary in their ability to form biofilms (Combrouse *et al.*, 2013; Fouladynezhad *et al.*, 2013).

2.3.2.3. Listeria monocytogenes Contamination in Ready to eat Foods

Listeriosis is most commonly caused by consuming contaminated food. *L. monocytogenes* has been isolated from many types of foods including raw and pasteurized milk, soft-ripened varieties of cheeses, ice cream, raw fruits and vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats of all types, and raw and smoked fish (Lianou and Sofos, 2007; Ryser and Marth, 2007).

In particular, RTE foods that support the growth of *L. monocytogenes* demonstrate the highest risk, since they are usually consumed without the cooking that can inactivate this pathogen (Todar, 2011). Examples of high risk RTE foods that support growth of *L. monocytogenes* include: Milk and dairy products (e.g. butter and cream); Soft unripened cheeses (e.g., queso fresco, cottage and ricotta cheese); Cooked crustaceans (e.g., shrimp and crab); Smoked seafood, smoked finfish and mollusks; Certain vegetables (e.g. cabbage, and non-acidic fruits

such as melons) and Some deli-type salad sandwiches (e.g. prepared from non-acidified seafood at retail establishments) (U.S. Department of Health and Human Services, Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2008).

Some RTE foods, such as fresh cut fruits and vegetables, may be naturally contaminated with *L. monocytogenes*. Some RTE foods such as seafood and deli meats are susceptible for post-process contamination at the food manufacturer, retail and at home. If refrigerated RTE foods that do not have any method to control growth of *Listeria* are contaminated with this pathogen, *L. monocytogenes* may grow to unsafe numbers in these RTE foods after prolonged storage, even if the initial contamination level is low (U.S. Department of Health and Human Services, Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2008).

2.3.2.4. Listeria monocytogenes Contaminations in raw milk

In milk production, it is impossible to produce sterile raw milk. Raw milk can become contaminated with *L. monocytogenes* through: Contaminated udders or teat canals; Mastitic animals; or Contaminated milking equipment, cleaning water, workers, and the environment (Leedom, 2006). The udders and teats of milking animals are contaminated with microorganisms when they come into contact with faeces, urine, feed (silage) soil, contaminated water and dirty equipment. The udders of housed cows can also become contaminated from contact with bedding materials (hay, sawdust). *Listeria monocytogenes* is environmentally ubiquitous but its presence can be enhanced on dairy farms through faecal shedding from animals fed poor quality silage or baleage. An outbreak of listeriosis among livestock will also create an opportunity for *L. monocytogenes* to multiply and be distributed in the farming environment (Leedom, 2006).

Listeria monocytogenes can be shed in the faecal matter of both clinically-infected animals and asymptomatic carriers. In asymptomatic animals, *L. monocytogenes* is unlikely to colonies the gastrointestinal tract for long periods of time. Rather, the pathogen either passé directly through the gastrointestinal tract without infecting the animals or colonize and multiplies in the gastrointestinal tract for short periods of time (2-4 days) (Ho *et al.*, 2007).

This leads to a pattern of intermittent faecal shedding with the animals being re-infected from feed or the farm environment.

Listeria monocytogenes is a natural contaminant in the plant material used for preparing silage and baleage and will survive and multiply in these foods if they are not fermented properly (Wiedmann and Evans, 2011). Feeding dairy animals with improperly fermented silage or baleage (pH ≥5.0) can introduce *L. monocytogenes* into herds and has been the cause of listeriosis outbreaks among livestock (Borucki *et al.*, 2005; Holmes and Brookes, 2006)

Mastitis caused by *L. monocytogenes* infection of the udder has been reported in dairy animals but it appears to be rare. The infection is more commonly reported in sheep and goats than in cows. Bovine mastitis caused by *L. monocytogenes* infection can go undetected as the cows do not necessarily demonstrate any outward signs of infection and the milk can remain visually unchanged (Hunt *et al.*, 2012).When considering only clinically mastitic animals, the proportion of infections caused by *L. monocytogenes* may be high. An Iranian survey found 8% (17/207) of clinically mastitic cows were infected with *L. monocytogenes* (Jamali and Radmehr, 2013). *Listerial* mastitis in just one animal can introduce a high number of *L. monocytogenes* cells into the bulk tank milk. *L. monocytogenes* was detected in bulk tank milk (Hunt *et al.*, 2012).

Listeria monocytogenes can also be transferred into milk from milking equipment that has become contaminated (through contact with the cows, milkers or contaminated water). Milk containing *L. monocytogenes* also effectively carries the pathogen into the milking system. *L. monocytogenes* are able to attach to the surfaces of milking equipment and the milking environment (drains) and create dense growths of living cells. Formation of these *L. monocytogenes* biofilms (a biofilm is a surface-associated three-dimensional community composed of differentiated strata and surrounded by an exopolymer substance (Moltz and Martin, 2005)) is influenced by the presence of other bacteria that are already attached to the surfaces (Flint *et al.*, 2011; Weiler *et al.*, 2013). The ability to form biofilms varies between *L. monocytogenes* strains and is not related to serotype (Weiler *et al.*, 2013).

Listeria monocytogenes can form dense growths within a short period of time and when these biofilms form in the milking equipment, cells can break away individually or as clumps and

contaminate raw milk. An in-depth analysis of *L. monocytogenes* strains present on a USA farm over several years provided evidence of persistent contamination in milk that probably arose from biofilm formation inside the milking equipment (Latorre *et al.*, 2009; Latorre *et al.*, 2010). *Listeria monocytogenes* strains that were persistent in the milk and milking equipment over time, one demonstrated strong adherence ability in vitro, supporting this strain's ability to form biofilms (Latorre *et al.*, 2011).

Listeria monocytogenes is able to attach to different surfaces by forming biofilms and consequently, it is able to spread and survive. Biofilm formation by *L. monocytogenes* is associated with its persistence in food environments and higher resistance to antimicrobials used in food environment. Periodically repeated surveys are needed for determining the prevalence and clones of *L. monocytogenes* in food manufacturing environment (Garrido *et al.*, 2009).

2.4. Pathogenesis and Virulence Factors

Listeria monocytogenes is a highly invasive intra-cellular pathogen. Macrophages actively ingest *L. monocytogenes.* It is this internalization of the bacterium that is triggered by *L. monocytogenes.* The pathogen appears in a vacuole, which is later lysed by the bacteria allowing it to escape in to the cytoplasm of the host cell. The bacterium then polymerizes actin filaments at one end to form long actin tails that propel it through the cytoplasm (Salyers and Whitt, 2002). The adherence and invasion are mediated by membrane proteins called internalins (InlA and InlB). Working together, InlA and InlB and several other bacterial surface proteins mediate internalization of *L. monocytogenes* into a single membrane vacuole (Olier, 2005; Longhi, 2008). To gain entry in the nutrient-rich cytoplasm, *L. monocytogenes* expresses a phospholipase PlcA and a cholesterol-dependant pore-forming hemolysin Listeriolysin O (LLO) which create holes in the single membrane (Pamer, 2004; Hamon *et al.*, 2006; Longhi, 2008).

Being a toxin, LLO must be tightly regulated to ensure only the vacuole membrane is destroyed. Over expression of LLO results in lysing of the host cell, consequentially exposing the bacterium to the host immune system (Hamon *et al.*, 2006). LLO has unique properties for

L. monocytogenes. LLO activity is activated by an acidic environment (pH 6) which is atypical of other cytolysins (Hamon *et al.*, 2006).

The forced phagocytosis brings *L. monocytogenes* into the host cells encased in a vacuole. The bacteria then escape the vesicle by a protein hemolysin, listeriolysin O (LLO) (Salyers and Whitt, 2002). LLO is a pore-forming cytotoxin. It is also responsible for the zone of beta-hemolysis seen on blood agar plates. The gene encoding LLO is named "hly" for "hemolysin."

To spread the infection to other cells, *L. monocytogenes* overtakes host cell machinery to induce actin polymerization with the help of bacterial surface protein ActA (Hamon *et al.*, 2006). This propulsion allows for undetected movement into neighboring cells intracellularly (Hamon *et al.*,2006). Pamer showed that infection with ActA-deficient bacteria is less pathogenic (Pamer, 2004). Entrance into a second cell creates a double-membrane around the bacteria. These layers are lysed by LLO and phospholipase PlcB. Thus, the bacteria enter the cytoplasm of the second cell and multiply again.

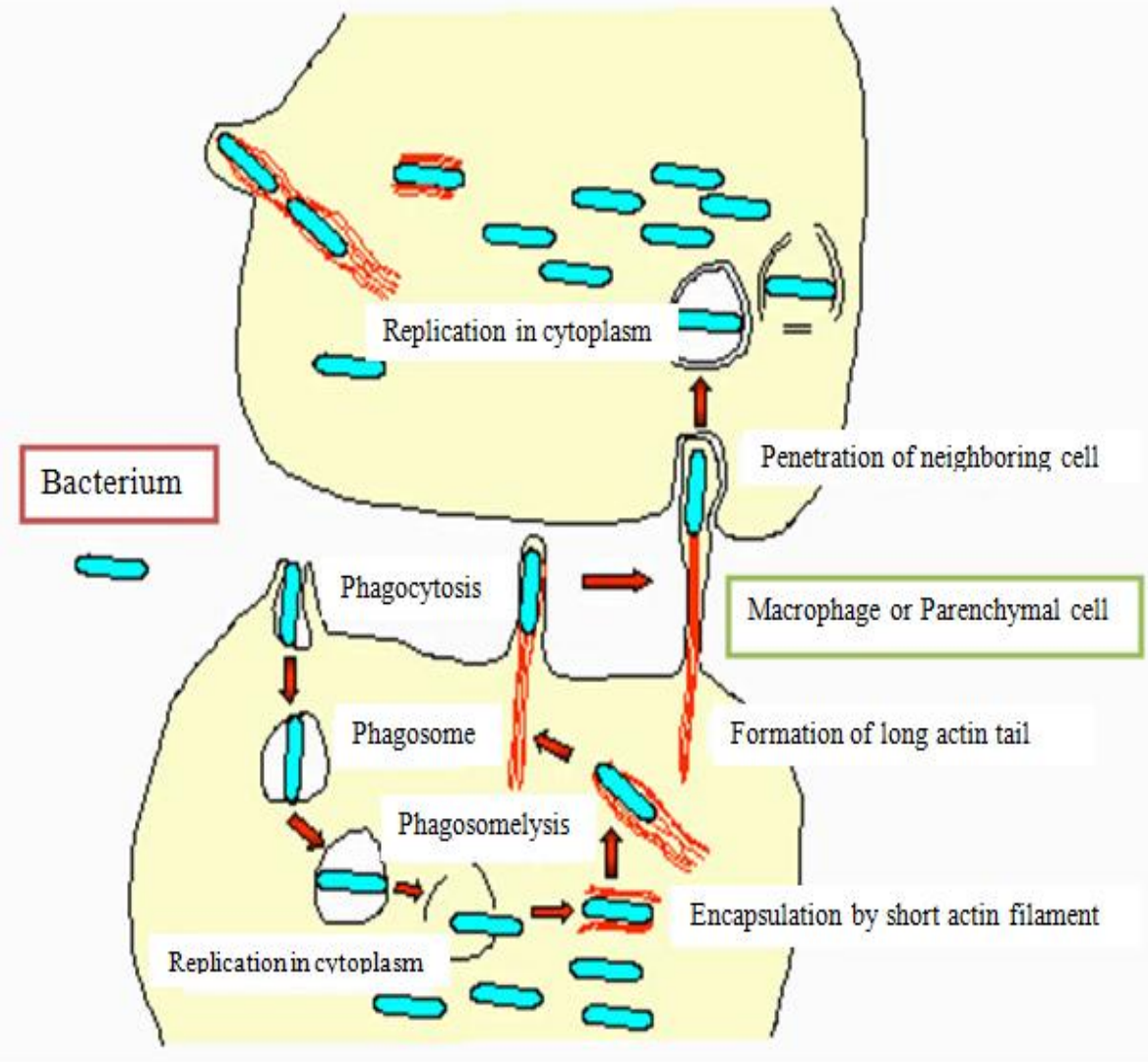

Figure 1**.** Cell to cell spread of *L. monocytogenes* by formation of actin filaments (act A gene)

Adapted from Todar's online textbook of Bacteriology (Todar, 2008)

Listeria monocytogenes is a facultative intracellular pathogen able to survive in macrophages and to invade a variety of non phagocytic cells, such as epithelial cells, hepatocytes and endothelial cells (Cossart *et al.*, 2003). When *L. monocytogenes* is ingested, it may survive the stomach environment and enter the intestine where it penetrates the intestinal epithelial cells. The organism is then taken up by macrophages and non-phagocytic cells. The *L. monocytogenes* surface protein internalin is required for this uptake by non-phagocytic cells, as it binds to the receptors on the host cells to instigate adhesion and internalization. The bacterium is initially located in a vacuole after uptake by a macrophage or non-phagocytic

cell. *L. monocytogenes* secrete listeriolysin O protein, which breaks down the vacuole wall and enables the bacteria to escape into the cytoplasm (Montville and Matthews, 2005; Todar; 2008).

Any bacteria remaining in the vacuole are destroyed by the host cell. Once located in the cytoplasm of the host cell, *L. monocytogenes* is able to replicate. *L. monocytogenes* is transported around the body by the blood, with most *L. monocytogenes* being inactivated when it reaches the spleen or liver. *L. monocytogenes* is able to utilize the actin molecules of the host to propel the bacteria into neighboring host cells. In the case of invasive listeriosis, this ability to spread between host cells enables *L. monocytogenes* to cross the blood-brain and placental barriers (Kuhn and Goebel, 2007; Bonazzi *et al.,* 2009).

2.4.1. Infective Dose

The minimum dose required to cause clinical infection in humans has not been determined but the large numbers of *L. monocytogenes* detected in food responsible for epidemic cases of listeriosis suggest that it is high, 10^6 Cfu/g. Nevertheless, low doses cannot be excluded in so far as they too may cause infection, especially in the high risk group. The levels of contamination as low as 10^2 or 10^3 Cfu/g have been linked with clinical cases. The infection dose may vary depending on the virulence of the strain and the host factors. Evidence comes from the fact that only 3 of the 13 known serotypes of *L. monocytogenes* (1/2a, 1/2b and 4b) are responsible for the majority of human and animal cases of listeriosis worldwide (Garrido *et al.,* 2008). However, serotypes 1/2a, 1/2b and 1/2c are the most frequently found in food products (Gombas *et al.,* 2003; Garrido *et al.,* 2009).

2.4.2. Symptoms

In a normal immuno-competent individual the infection is usually asymptomatic or with mild influenza-like symptoms, it can be carried with or without apparent symptoms, up to 5-10% in the intestinal tract of humans. However, in susceptible individuals like; younger, older, pregnant women and immuno-compromised (YOPI) individuals (people with Cancer, HIV, rheumatoid disease, diabetes, cirrhosis of the liver, asthma), illness is likely to occur with serious (overt) form of the disease. In Europe, North America, Japan and Australia the proportion of elderly people (>65 years of age) expected to double with the next 30-35 years

and in countries like Germany and Italy this proportion approaches to about 30% (www.bfhd.wa.gov, 2003).

The form of listeriosis frequently reported in adults is that affects the CNS (55 to 70% of cases) as meningo-encephalitis. However, the encephalitis form, in which *Listeria* organisms are isolated with difficulty from the cerebrospinal fluid, is common in animals but rare in humans (Vazquez-Boland *et al* ., 2001).

It has been estimated that *L. monocytogenes* accounts for 10% of commonly-acquired bacterial meningitis. Due to effective vaccination against H. influenza, *L. monocytogenes* is now the 4th most common cause of meningial infection in adults after Streptococcus pneumoniae, N. meningitidis and group B Streptococci (FDA and CDC, 2003).

There are other atypical clinical forms (5 to 10%) of cases such as endocarditis, (the 3rd most frequent form), myocarditis, arteritis, pneumonia, pleuritis, hepatitis colecystitis, peritonitis, localized abscesses, (example brain abscess, which accounts for about 10% of CNS infection by *Listeria* spp.) arthritis, osteomyelitis, sinusitis, otitis, conjunctivitis and ophthalmitis There is also a primary infection mostly seen in people associated with animals like veterinarians and farmers characterized by a pyogranulomatous rash (Vazquez-Boland *et al* ., 2001).

In pregnant women infection of the fetus is extremely common and can lead to abortion, still birth or delivery of *Listeria* infected infant. Granulomatosis infantiseptica is one of the syndromes, which is associated with fetomaternal or neonatal form of meningitis and is characterized by the presence of pyogranulomatous abscesses disseminated over the body. The neonatal form of infection is reclassified as early- onset of sepsis, which results in premature birth and late onset meningitis, which is acquired through vaginal transmission. Of all pregnancy-related cases, 22% result in fetal loss or neonatal death, but mothers usually survive. Corticosteroid therapy is the most important predisposing factor in patients who are not pregnant. CNS infection in general, is very rare during pregnancy, although it is observed frequently in other compromised hosts. For example, brain abscess occurs in 10% of CNS infections, often located in the thalamus, Pons, and medulla. This unusual complication is associated with high mortality (Vazquez-Boland *et al.*, 2001).

In food infection, febrile gastroenteritis syndrome is the main clinical manifestation of *L. monocytogenes* infection. The potential entero-pathogenicity of *L. monocytogenes* has also been recognized in animals with outbreak of diarrhea and gastroenteritis having been reported in sheep. Another major clinical manifestation form of listeriosis, occurred in ruminants especially sheep (animals), is meningo-encephalitis also known as a 'circling disease'. It was first described by Gill in 1933 in New Zealand. The syndrome is characterized by involuntary torticollis and walking aimlessly in circles as a result of brain stem lesions. In its severe form, animals lie on the ground with evident signs of un-coordination (paddling movement) and cranial nerve paralysis (strabismus, salivation). In cows, it causes mastitis (Ryser and Donnelly, 2001).

2.5. Laboratory isolation methods

2.5.1. Culture method

A number of methods are available for the detection of *Listeria* species. However, culture is the best method, with detection power of about 10^2 Cfu/ml. Colonies of *Listeria* species in general are small, smooth, translucent, and bluish gray when viewed in normal light, but blue green sheen is visible by oblique light. The most widely used selective media is OXA (Oxford agar) agar which was developed in 1989. Most literatures recommended to use OXA agar and either one of the following media; PALCAM (Polymixin Acriflavine, Lithium chloride, Ceftazidime Aesculine Mannitol), MOX (Modified Oxford Agar), and LPM (Lithium Chloride Phenylethanol Moxacalatam medium) with esculine and ferric iron (Kiiyukia, 2003).

The principle of the media is the ability of esculine hydrolysis by *Listeria* species. Since all *Listeria* species utilize B-D-Glucosidase, they cleaved esculine (esculinase), evidenced by blackening of the medium. In addition, different antimicrobial agents like acriflavin, nalidixic acid, cyclohexamide are supplemented with those media to suppress the growth of other non-*Listeria* species of bacteria and other microbial agents. Various conventional methods are known for the isolation of *L. monocytogenes* and other *Listeria* spp. from food and clinical samples that have gained acceptance for international regulatory purposes. The tolerance limit of *L. monocytogenes* differs from country to country and also based on the food type. For instance, the USA requires absence of *L. monocytogenes* in 25 g of ready to eat foods (Zero

tolerant) while some European countries have a tolerance level of 100 Cfu/ml of *L. monocytogenes*/ml (FDA and CDC, 2003).

Table 4. Types of methods and their choice of media used for *Listeria* species identifications.

Type of the method	1^0 selective enrichment used	2^0 selective enrichment used	Selective media
The United States Food and Drug Administration, Center for Food Safety and Applied Nutrition (FDA-CFSAN) method,	*Listeria* enrichment broth (LEB)	Non/ LEB II	lithium chloride-phenyl ethanol-moxalactam medium (LPM) and Oxford agar plates
The Association of Official Analytical Chemists (AOAC) official method,	Trypticase Soy broth with 0.6% yeast extrract	-	PALCAM
The International Organization for Standardization (ISO11290)	half Fraser broth	Fraser broth	PALCAM and Oxford agar
The International Dairy Federation (IDF) method,	Modified *Listeria* enrichment both (LEB)	Either non/ Fraser broth	PALCAM and/or Oxford
The United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) method,	University of Vermont medium (UVMI)	either UVM II or Fraser	lithium chloride-phenyl ethanol-moxalactam broth medium (LPM)
The French Standards (Association Francaise de Normalization, AFNOR) method	Demi-Frase broth	Fraser broth	Oxford or PALCAM
Health Canada method for foods and environmental samples	*Listeria* enrichment broth (LEB)	Modified Fraser broth	Oxford agar, lithium chloridephenylethanol-moxalactam (LPM), modified Oxford/ PALCAM

Sources: (Manual, 2004)

2.5.2. Biochemical test

Biochemical tests are used for identification and confirmation of *Listeria* species, following culture isolation. The following four major media are used for this purpose. These are carbohydrate fermentation tests; rhamnose, D-xylose, mannitol and haemolysis on blood agar. In general, *L. monocytogenes* is catalase positive, glucose fermenter with acid production but no gas formation, ferment lactose (after 3-5 days of incubation), urease negative, oxidase negative, methyl red and Voges Proskauer positive, indole test negative, express hemolysis which produces clear zone on blood agar (Beta hemolytic), do not utilize citrate, do not produce hydrogen sulfide and also do not hydrolyze urea, gelatin, and casein (Vazquez-Boland *et al.*, 2001; Ryser and Donnelly, 2001).

Table 5. Biochemical chart for *Listeria* species identification

Species	Production of acid			Hemolysis	CAMP test	
	Xylose	Rhamnose	Mannitol		S.aureus	R.equi
L. monocytogenes	-	+	-	+	+	-
L .innocua	-	+/-	-	-	-	-
L .ivanovii	+	-	-	+	-	+
L. seeligeri	+	-	-	(+)	(+)	-
L. welshimeri	+	+/-	-	-	-	-
L. Grayi	-	-	+	-	-	-
L. Murrayi	-	+	+	-	-	-

+/-: variable; (+): weak reaction; +: >90% positive reaction; -: no reaction (-); R. equi; Rhodoccocus equi. Sources: (OIE Terrestrial Manual, 2014)

2.5.3. Chromogenic/ Rapid test

Rapid detection methods are available by using several methods such as PCR (Polymerase chain reaction), monoclonal antibodies and nucleic acid hybridization tests, and fluorogenic tests. In addition, alternative chromogenic differential selective agars like BCM, ALOA, CHROM agar *Listeria* and rapid *L. monocytogenes* can be used in parallel with one of the selective agars (Cox *et al.*, 1998). FDA recommended and validated such chromogenic rapid

kits with threshold of detection greater than 10^4 Cfu/ml of enrichment culture and incubation period of 4-6 hours. The advantage of such tests, are their principle lies on identifying of a specific virulence factors like phosphatidylinositlol specific phospolipase and in enumeration of the organism in the food samples (FDA and CFSAN, 2003).

2.5.4 Serological test.

If possible, all culture isolates of *Listeria* species should be typed serologically and genetically according to FDA rules (FDA and CFSAN, 2003), but it is expensive. Such serotyping is performed by mixing commercial sera and *Listeria* species suspending in fluorescent antibody buffer, after several washing and pelleting steps, in order to observe the positive clotting/precipitating reactions. Usually serological classification is done only in reference laboratories and is primarily used for epidemiological studies. These methods target to detect flagella or somatic sub-factors. Most *Listeria* species have different serotypes in which they share one or more antigens with *L. monocytogenes*. The following serotypes are exhibited within the species and also known as serotypes of *L. monocytogenes*; these are 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Of this 1/2a, 1/2b, and 4b are mostly isolated from patients and environment that account for 92% of the isolates among humans and animals and 4b is associated with cheese made from un-pasteurized milk (Doumith *et al.*, 2004). There is a significant geographical variation and 4b is identified as the predominant serotype in Canada, USA and Europe. Fifty percent of the sporadic infection outbreaks have been determined as serotype 4b (Doumith *et al.*, 2004).

2.6. Listeriosis in animals

Listeriosis has been detected in nearly all domestic animals (Pal, 2007). Most listeriosis cases have been reported in sheep, among which L. ivanovii is also a significant cause of *Listeric* infections, and also cows and goats, causing encephalitis, abortion, or septicemia (Wesley *et al.*, 2002). In sheep and cows, subclinical mastitis and gastroenteritis caused by *L. monocytogenes* have also been reported (Clark *et al.*, 2004; Rawool *et al.*, 2007).

In monogastric animals, listeriosis is rare, and large epidemics with generalized listeriosis and acute deaths have been reported only in farmed chinchillas (Wesley, 2007). In swine, the primary manifestation of listeriosis is septicemia, whereas in horses, abortions and

encephalitis is also typical (Wesley, 2007). Listeriosis of fowls is probably secondary to viral infections, and typically causes septicemia with accompanying cardiac lesions (Wesley, 2007).

Ruminants and poultry are known to be an important source of human listeriosis. The prevalence of *L. monocytogenes* at cattle farms with a history of *L. monocytogenes* infection was 24.4%, which was higher than data from farms without any history (20.2%) from New York state, USA (Nightingale *et al.*, 2004). In northern Spain, *L. monocytogenes* was identified from 46.3% of dairy cattle and 30.6% of beef cattle (Esteban *et al.*, 2009). At slaughterhouse, the mean prevalence data of *L. monocytogenes* were faeces with 19% (4.8–29%), hides 12% (10-13%), and raw beef products 10% (1.6–24%) (Rhoades *et al.*, 2009). In Jordan, *L. monocytogenes* was found in beef (19.2%), whereas *L. innocua* and *L. welshineri* were identified in 24% and 14.2% of samples, respectively (Awaisheh, 2010).

Listeria can infect poultry, human beings predominantly gets infection from raw broiler meat due to contaminations and unhygienic conditions of the processing area, rather than acquiring direct infection from birds (Goh *et al.*, 2012). The detection rate of *L. monocytogenes* from raw chicken samples in Turkey was 18% (Akpolat *et al.*, 2004). In Jordan, *L. monocytogenes* was found in 15% of poultry, while *L. innocua* and *L. welshineri* were identified in 23% and 15.8% of the samples, respectively (Awaisheh, 2010).

In livestock, listeriosis is associated with indoor housing and consumption of bad quality feed, especially silage (Wesley, 2007). The sensitivity of pregnant animals to listeriosis has led to epidemics in which the only symptoms were abortions. Symptomless faecal carriage of *L. monocytogenes* has been reported in primates, other mammals, and birds (Hellström *et al.*, 2008; Esteban *et al.*, 2009). The highest prevalence, up to 30%, has been reported in cattle followed by other ruminants, whereas companion animals seldom carry this species (Lyautey *et al.*, 2007; Esteban *et al.*, 2009).

2.7. Antimicrobial susceptibility pattern

Many antibacterial drugs inhibit *Listeria* in vitro. Some literatures reported that, *L. monocytogenes* is susceptible to wide range of antibiotics like ampicillin, erythromycin,

kanamycin, streptomycin, nalidixic acid and others except for cephalosporin and fosomycin/ fluoroquino lones (Williams, 2005: Hansen *et al.,* 2005; Aarestrup *et al.,* 2007). However, it has been reported that resistant *L. monocytogenes* strains were found frequently from time to time, even to common conventional drugs like clindamycin, sulfomethoxazole (Shen *et al.,* 2006) and also to enrofloxacin (Antunes *et al.,* 2002). Plasmid-borne resistance to chloramphenicol, macrolides, and tetracycline has also been identified (FDA and CFSAN, 2003).

The first choice of therapy is ampicillin combined with different amino-glycosides like gentamicin, TMP/SMX (Trimethoprim/ sulfomethoxazole) is recommended as the second choice of therapy or as alternative agent. However, other agents are mostly reported as static, and not cidal. On the other hand, the antimicrobial product activity of some aquatic bacteria, were also tested, which showed good inhibitory ability (Motta *et al.,* 2004).

2.8. Treatment and Prevention

Antibiotic therapy is the treatment of choice in most of the complications and the dose and duration of the treatment differ accordingly. For instance, bacteriamia should be treated for 2 weeks, if the patient is immuno-competent. However, longer courses may be required in the immuno-compromised patient. Similarly, meningitis should be treated for 3 weeks; while endocarditis for 4-6 weeks; and brain abscess for a minimum of 6 weeks. Ampicillin without or with gentamycin is generally considered as the preferred agent but other effective agents like co-trimaxazole can be used for empirical antimicrobial treatment (Hansen *et al*., 2005).

Preventive measures have to target the organism's nature (conditions for its normal growth) in addition to good sanitation and adequate heat treatment of food before consuming. Moreover, its growth (doubling time) is highly dependent on the temperature, pH, type of the food sample and background of the microflora present (Morrow *et al.,* 2004).

Listeria monocytogenes can survive and multiply within the range of 1°C to 45°C. There are large strain-to-strain variations but some strains seem to be able to grow down to about -1.5°C. The relationship between temperature and *L. monocytogenes* rate of multiplication was studied and reported that the colder the temperature, the slower rate of multiplication was

observed (Johan *et al.,* 2004). Lactate and diacetate are effective against *L. monocytogenes* contamination in cured ready-to-eat meat products, and also reported about effectiveness of anti-mycotic agents such as benzoate, sorbate, pediocin and propionate in combination with nitrite in inhibiting *Listerial* growth in ready-to-eat meat and poultry products (Katla *et al.*, 2003). The growth of *L. monocytogenes* can be prevented, or at least reduced, by other means, for example by reducing the pH or by increasing the salt content (Morrow *et al.,* 2004).

2.9. Status of Listeriosis in Ethiopia

In Ethiopia only a few studies were undertaken on the status of *L. monocytogenes* and other species. According to Molla *et al.,* (2004) of a total of 316 food samples analyzed, 103(32.6%) were positive for *Listeria* species .In this study the level of contamination of food samples, by *Listeria* species was the highest in pork (69.8%) and followed by minced beef (47.5%) and ice cream (43.5%). The dominant *Listeria* species was *L. innocua* (21.2%) followed by *L. monocytogenes* (5.1%). Another study under taken by Mengesha (2005), from a total of 711 different food samples analyzed for the presence of *Listeria* species, 189(26.6%) of the food samples were found to be positive for *Listeria* species with the dominant species being *L. innocua* (17.7%) and followed by *L. monocytogenes* (4.8%).

According to Abera (2007), study in Addis Ababa from a total of 240 different food samples (cottage cheese, cream cake, raw milk and raw meat) examined 66(27%) were positive for *Listeria* species. The levels of contamination of *Listeria* species was higher 41(17%) in raw meat samples followed by cream cake 13(5.4%), raw milk 6(2.5%) and cottage cheese 6(2.5%). In this studies the dominant *Listerial* species isolated was *L. innocua* 26(10.8%) followed by *L. monocytogenes* 13(5.4%), *L. murrayi* 9(3.7%) and *L.welshimeri* 8(3.3%).

Another study under taken in Addis Ababa by Gebretsadik *et al.* (2011), from a total of 391 different food samples examined 102(26.1%) were found to be positive for *Listeria* species. The levels of contamination of *Listeria* species was higher in raw beef 39(51.3%) followed by liquid whole egg 37(32.2%), raw milk 22(22%) and cottage cheese 4(4%). In this study the dominant *Listeria* species isolation was *L. innocua* (60.8%), *L.* welshimeri (6.9%), *L. monocytogenes* (5.4%), *L. seeligeri* (3.9%), *L. murrayi* (2.9%), *L. grayi* (2.9%) and *L. ivanovii* (1.9%).

Studies under taken in Jimma town by Muhammed *et al.* (2013), from total of 200 milk and milk product samples (milk, cottage cheese, ice cream and yoghurt) examined and found that 13(6.5%) were positive for *Listeria* species. In this study the level of contamination of *Listeria* species was higher 7 (14%) in milk samples followed by ice cream 3(6%), yoghurt 2(4%) and cottage cheese 1(2%). The dominant *Listeria* species isolated was *L. monocytogenes* 8(4%) followed by *L. innocua* 3(1.5%) and *L. seeligeri* 2(1%). Recent study's under taken in Gondar town by Garedew *et al.* (2015), a total of 384 food samples examined, 96 (25%) were positive for *Listeria* species.

According to this study the highest and the lowest prevalence of *Listeria* were found in raw meat 31(51.66%) and cottage cheese 5(12.5%) respectively. All pasteurized milk (n= 50) samples examined were negative for *Listeria* species. *L. monocytogenes* were isolated from 24(6.25%) of the total food samples analyzed and it was the second predominant *Listeria* species next to *L. innocua* (12.5%). The prevalence of *L. innocua* was found to be the highest in raw meat (31.7%) followed by raw milk (14%) and fish meat (10%) samples. In this study, ice cream was the most contaminated food item (15%) with *L. monocytogenes* followed by minced beef (12%) and cream cakes (10.7%) (As shown in Table 6).

Table 6. Prevalence of *L. monocytogenes* and other *Listeria* species from food samples in Ethiopia

Authors	**Place of study**	**Types of food samples**	**No. of Sample Examined**	**No. of positives *L. monocytogene***	**No.positives other *Listeria* sps**	**Total No. of positive (%)**
Molla *et al.* (2004)	Addis Ababa	Minc. Beef.	61	1(1.6%)	28(45.9%)	29(47.5%)
		Chicken	52	1(1.9%)	7 (13.5%)	8(15.4%)
		Cott. cheese	61	-	1 (1.6%)	1(1.6%)
		Fish	43	1(2.3%)	7 (16.3%)	8(18.6%)
		Ice cream	46	9(19.6%)	11 (20.7%)	20(43.5%)
		Pork	53	4(7.5%)	33 (62.3%)	37(69.8%)
		Total	**316**	**16 (15.5%)**	**87 (27.5%)**	**103(32.6%)**
Abera, (2007)	Addis Ababa	Cott. cheese	60	0	6(10%)	6(10%)
		cream cake	60	2(3.3%)	11(18.3%)	13(%)
		Raw milk	60	0	6(10%)	6(10%)
		Raw meat	60	11(18.3%)	30(50%)	41(68.3%)
		Total	**240**	**13(5.4%)**	**53(22%)**	**66(27.5%)**
Gebretsadik *et al.* (2011)	Addis Ababa	Cott. cheese	100	1(1%)	3 (3%)	4(4%)
		Raw beef	76	2(2.6%)	37 (48.7%)	39(51.3%)
		Raw milk	100	13(13%)	9 (9%)	22(22%)
		whole egg	115	5(4.3%)	32 (27.8%)	37(32.2%)
		Total	**391**	**21 (5.4%)**	**81 (20.7%)**	**102(26%)**
Muhammed *et al.* (2013)	Jimma Town	Milk	50	5(10%)	2(4%)	7(14%)
		Cott. cheese	50	0	1 (2%)	1(2%)
		Ice cream	50	2(4%)	1(2%)	3(6%)
		Yoghurt	50	1(2%)	1 (2%)	2(4%)
		Total	**200**	**8 (4%)**	**5(2.5%)**	**13(6.5%)**
Garedew *et al.* (2015)	Gondar Town	Raw meat	60	4(6.66%)	27(45%)	31(51.7%)
		Minc. beef	25	3(12%)	3(12%)	6(24%)
		Fish meat	50	3(6%)	10(20%)	13(26.0%)
		Pizza	24	2(8.3%)	5(20.8%)	7(29.2%)
		Past. milk	50	-	-	-
		Raw milk	50	2(4.0%)	12(24%)	14(25.0%)
		Cott. cheese	40	0	5(12.5%)	5(12.5%)
		Ice cream	20	3(15%)	6(30%)	9(45%)
		Cr. Cake	65	7(10.7%)	4(6.15%)	11(16.9%)
		Total	**384**	**24(6.25%)**	**72(18.7%)**	**96(25%)**

Minc= minced, cott. = cottage, past= pasteurized, Cr. Cake = Cream Cake

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Debre-birhan town of Amhara Regional State; North Shoa, Ethiopia. The Town is located 130 km away from the capital city (Addis Ababa) at $9^0$41' N latitude and $39^0$32' E longitude and the elevation is ranged from 2700 to 2800 meter above sea level. The area receives annual rain fall ranges from 814 to 1080 mm with about 70% rain falling between June and September. The annual average temperature of the town ranges between 4^0C in the coldest month (August) to 26°C in the hottest month (April) which is categorized under "dega" or tropical climate weather condition. The town has 23,853 cattle, 27,580 sheep, 2,400 goat, 1,380 horse, 52 mule, 3,238 donkey, 59,470 poultry and 235 honey bee colonies. From the total cattle populations, 9,287 cattle are exotic breed and 14,566 are local breeds. 20 Milk collection centers are found in the town (North Shoa Zone, Urban Agriculture office data, 2013).

3.2. Study design

A cross-sectional study was conducted at Debre-birhan town from January to September 2015, to determine the prevalence of *L. monocytogenes* and other *Listeria* species, and also the antimicrobial susceptibility profile of *L. monocytogenes.*

3.3. Sample size determination

Since there was no previous study in the area on raw milks from dairy producers, sample size was estimated by taking 50% expected prevalence with 95% confidence interval and 5% desired absolute precision (Thrusfield, 2007).

$$n = \frac{(Z\ \alpha/2)^2\ (P_{exp.}(1-P_{exp.})}{d^2}$$, where; n = is the minimum sample size

Pexp. =Expected prevalence

Z α/2 =1.96 at 95% confidence interval.

d = desire absolute precision

Accordingly a total of 384 dairy producers were included in this study.In addition, 23 venders were also sampled for the detections of *L. monocytogenes.*

3.4. Sampling method and collection

Study samples were selected using simple random sampling technique and 384 dairy producers that deliver their milk to 13 milk collection centers were sampled. All dairy producers encountered in this study were smallholder dairy farms having one to five, and some are more than five lactating dairy cows. Each raw milk samples were collected in sterile snap-cap milk (sterile plastic containers). 30 ml of raw milk were collected aseptically in sterile plastic containers according to Jorgensen *et al.* (2005) from milk containers of the dairy producers at the sites of milk collection centers. 23 milk samples from bulk milk container of vendor's were also taken. The collected samples were identified by date of collection, code of the sample and their source. All milk samples were transported immediately by using icebox to Debre- birhan agricultural research center microbiology laboratory and stored at 4^0 C until analysis.

3.5. Laboratory activities

3.5.1. Isolation and identifications of *Listeria* species

Bacteriological examination was done to isolate *L. monocytogenes* and other species of *Listeria* from raw milk samples. For the isolations and identifications of *Listeria* species in food samples, the techniques recommended by the International Standards Organization (ISO 11290-1, 1996) and the French Association for Standardization (AFNOR, 1993) were employed. Oxford agar was used for selective plating and identification of *Listeria* species.

3.5.2. Sample Preparation

Each sample unit was agitated thoroughly to ensure the homogeneity of its contents and obtained aseptically by taking directly 30ml raw milk sample unit into Erlenmeyer flask. Then, the samples were thoroughly mixed by agitating the raw milk.

3.5.3. Primary and secondary selective enrichment

Twenty five milliliters of each sample from the composite mixture was measured and transferred into 225 ml of previously prepared half Fraser broth followed by mixing the suspended sample for two minutes using a laboratory blender at 2400 rev/min. Finally the samples were incubated at 30^0 C for 24 hrs.

After 24 hrs of incubation, the inoculated half Fraser suspension was mixed by swirling the Erlenmeyer flask and 0.1 ml was drawn and inoculated into 10 ml of Fraser broth and then incubated for 48 hrs at 35^0 C. Growth in the Frazer broth was very apparent, with colour change from the initial golden yellow to dark green or black; unlike in the half Fraser which has no showed colour change (Figure 2 and 3)

Figure 2. Sample inoculated Half Fraser broths no color changes showed

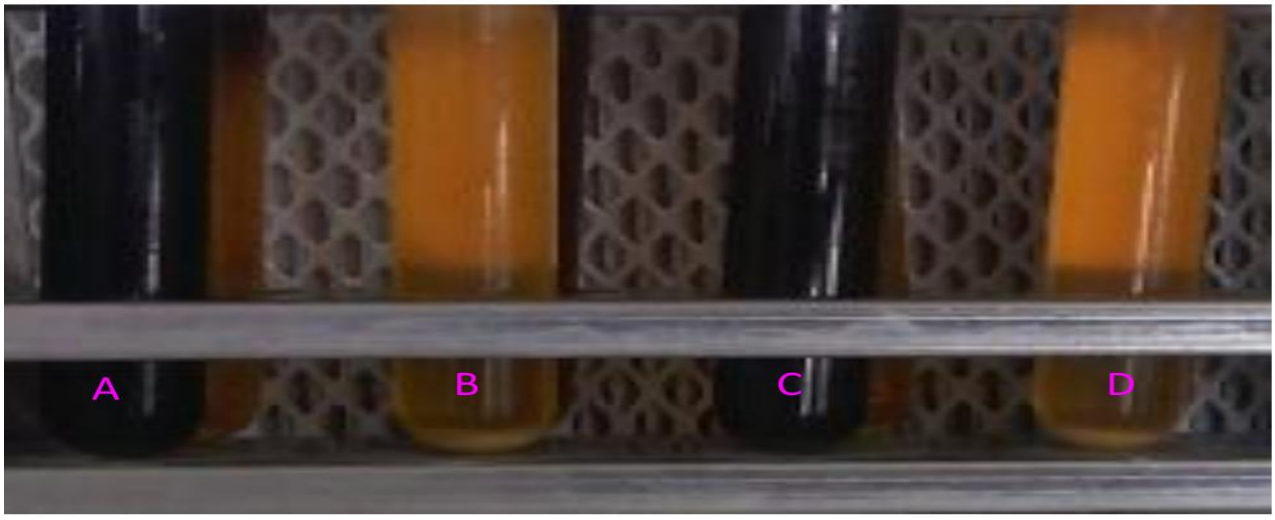

Figure 3. Color changes after enrichment in Fraser broth for samples of raw milk.
A) Positive control for *Listeria monocytogenes* (ATCC 11911), B) Negative samples for *Listeria*, C) Positives for *Listeria* species, D) Negative control or no samples inoculated.

3.5.4. Isolation

As a continuation of the procedure from the selective enrichment broth, a positive Fraser broth media with black /dark brown or dark green color was inoculated by taking a loop-full

of the suspension into selective media Oxford agar (OXA) and the plate were incubated at 37^0 C for 48 hrs. Growth of 1-2 mm diameter black or black green colony with a black halo and black sunken center was taken as positive for *Listeria* species. The colonies of *Listeria* were identified as per Falana (Falana *et al.*, 2003) on Oxford agar colonies appeared brown black or greenish black with a depressed center and a surrounding black halo(Figure 4).

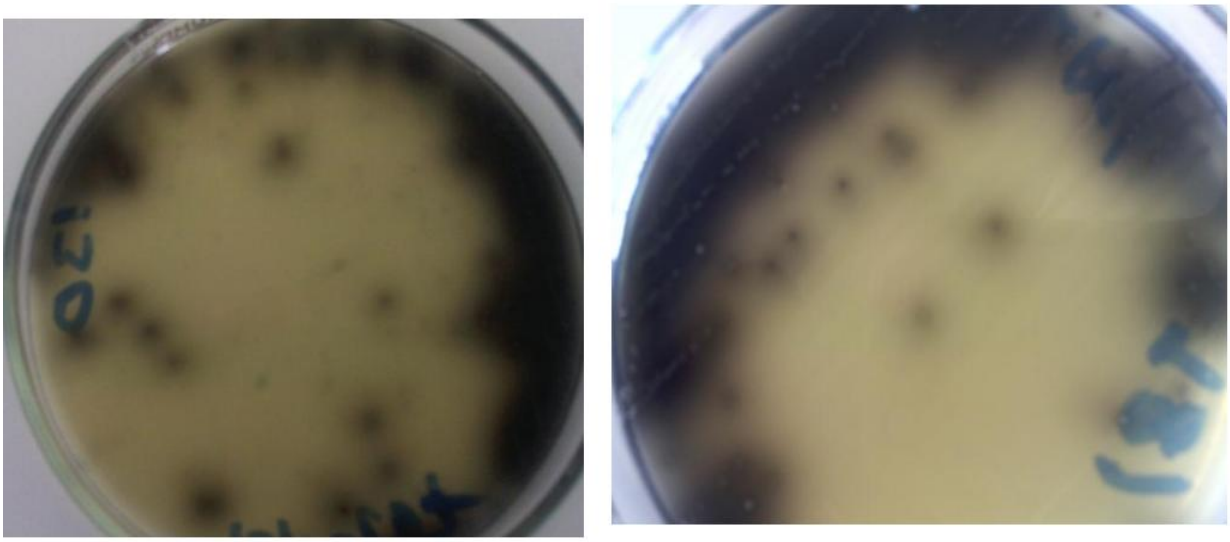

Figure 4. Colonies of *Listeria* species on Oxford (OXA) Agar

When the colonies were grown and well isolated on the selective media, about 3-5 colonies from each medium was picked and inoculated into pre dried plates of Tryptone Soya Yeast Extract Agar and incubated for 18 to 24 hrs at 37^0 C to obtain pure culture. Any grey-whitish colony was suspected to be positive for *Listeria*. Typical colonies (1 mm to 2 mm in diameter, convex, colorless and opaque), were used for further Gram staining, Catalase test and biochemical tests.

Gram-staining

The Gram-stain was performed on a colony separated in Tryptone Soya Yeast Extract Agar (TSYEA). *Listeria* species were revealed as Gram positive, slim, short rods.

Catalase reaction

An isolated colony obtained from Tryptone Soya Yeast Extract Agar were taken and suspended in to a drop of 3% of hydrogen peroxide solution on a slide. The immediate formation of gas bubbles indicated as a positive reaction.

3.5.5. Biochemical and other confirmatory tests

The *Listerial* isolates were then confirmed and also for specification, different standard biochemical tests were used. The biochemical confirmatory tests were done by picking pure colonies and transferring into the following biochemical media and broths. These were: motility test medium (motility), blood agar (haemolysis), mannitol, rhamnose and xylose broths for carbohydrate fermentation testing. CAMP test was also done by streaking standard strain of S. aureus (ATCC 25923) vertically on the blood agar and streaking *Listeria* isolates horizontally to S. aureus streak (Lovett *et al.,* 1989).

Motility test

Isolated colonies obtained from Tryptone Soya Yeast Extract Agar were taken and suspended in a tube containing Tryptone Soya Yeast Extract Broth (TSYEB) and incubated at 25^{o}C for 8 h to 24 h until a cloudy medium is observed. A drop of the above culture was deposited using a loop on to a clean glass microscope slide. A cover slip was placed on the top and examined under the microscope. *Listeria* species appeared as slim, short rods with tumbling motility. Cultures grown above 25^{o}C may fail to exhibit this motion. The *Listeria* species are motile, giving a typical umbrella like growth pattern.

Haemolysis test

When morphological and physiological characteristics and catalase reaction were indicative of the *Listeria* species, 5% sheep blood agar plates were inoculated to determine the hemolytic reaction. Colonies separated from Tryptone Soya Yeast Extract Agar were streaked one space apart from each culture, using a loop. Simultaneously positive (*L. monocytogenes*) was also streaked. After incubation at 37^{o}C for 24h±2h, the test strains and controls were examined. *L. monocytogenes* showed narrow, clear light zones of β-haemolysis. Plates were examined in a bright light to compare test cultures with controls.

Carbohydrate utilization test

Each of the carbohydrate broths (rhamnose, mannitol and xylose) was inoculated with a culture from TSYEB and was incubated at 37^{o}C for up to 5 days. A positive reaction (acid formation) was indicated by yellow color production and occurred mostly within 24h to 48h. *L. monocytogenes* are shown acid production only in rhaminose but not in both mannitol and

xylose. *L. grayi* and *L. murrayi* are shown acid productions in mannitole. While *L. ivanovii*, *L. seeligeri* and *L. welshimeri* have shown acid productions in xylose. *L. innocua* and *L. welshimeri* have variably (+/-) shown acid production in Rhamnoses. However *L. grayi*, *L. murrayi* and *L. innocua* show no acid production in xylose. *L. murrayi* has shown acid production in Rhamnose.

CAMP Test: (Christie, Atkins, Munch, Petersen test)

The CAMP test is a useful tool to identify the species of a *L. monocytogene* isolates; the test was undertaken using standard strain of *Staphylococcus aureus* (*S. aureus*) (American type culture collection (ATCC) 25923) by streaking in a single line across the sheep blood agar plate and the test strains separated were streaked in a similar fashion at right angles to *S. aureus*. Simultaneously, control cultures of *L. monocytogenes* (ATCC 11911) (kindly obtained from the Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia) were streaked on to sheep blood agar plates. The test culture and control culture do not touch *S. aureus* cultures but at their closest and are about 1 to 2 mm apart. The plates were incubated at 37^{o}C for 18 h to 24 h. An enhanced zone of β-haemolysis at the intersection of the test strain with each of the cultures of *S.aureus* were considered to be a positive reaction. A positive reaction with *S. aureus* appears as a small zone of enhanced haemolysis extending only about 2 mm from the test strain and within hemolytic zone due to growth of the *S. aureus* culture.

Table 7. Differentiations of main *Listeria* species

Species	Production of acid from			Hemolysis	CAMP test
	Xylose	Rhamnose	Mannitol		S.aureus
L. monocytogenes	-	+	-	+	+
L .innocua	-	+/-	-	-	-
L .ivanovii	+	-	-	+	-
L. seeligeri	+	-	-	(+)	(+)
L. welshimeri	+	+/-	-	-	-
L. Grayi	-	-	+	-	-
L. Murrayi	-	+	+	-	-

+/-: variable; (+): weak reaction; +: > positive reaction; -: no reaction. Sources: (OIE Terrestrial Manual, 2014)

3.5.6. Antimicrobial susceptibility test

Antimicrobial susceptibility test were performed for *L. monocytogenes* isolates by using Muller Hinton Agar. The common conventional antimicrobial drugs (cephalotin, Nalidixic acid, chloramphenicol, kanamycin, steptomycin, tetracycline, vancomycin, gentamicin and Ampcillin) were tested.

The method applied for antimicrobial testing was agar plate antibiotic disk diffusion method by adjusting to 0.5 McFarland turbidity standards (Antunes *et al.*, 2002; Hansen *et al.*, 2005). About 2-3 pure colonies of the isolates were taken from the Tryptone Soya Yeast Extract Agar and suspended in Muller Hinton broth and then, incubated at 37^0 C for 1-2 hrs. The suspension was checked for the development of slight turbidity. It was inoculated to Mueller-Hinton agar plate by using a sterile cotton swab. Plates were seeded uniformly by rubbing the swab against the entire agar surface. After the inoculums were dried, antibiotics impregnated disc were firmly placed on the surface of the inoculated plates. The plates were incubating at 37^0 C for 24 hrs. After incubation the zones of inhibition around each disc were measured and the results were interpretate as sensitive, intermediate and resistant using a standard zone interpretative chart (NCCLS, 2004) (Annex 8).

3.5.7. Quality control

The correct performance of all stages of the analysis, including enrichment, screening tests, plating and all confirmatory tests were verified through the use of *L. monocytogenes* (ATCC 11911) reference strains.

3.6. Data Management and Analysis

Microsoft Excel was utilized for data entry and storage and analysis were done by using statistical package for social science (SPSS 2007, version 16). Descriptive statistics were used to describe and process the data. Comparisons between detection rates of groups were analysed by using Chi square (χ^2). For alltests, p-value less than 0.05 were considered to be significant.

4. RESULTS

4.1. Detection of *L. Monocytogenes* and other *Listeria* species in raw milk samples from Dairy producers and Vendors.

A total of 407 samples of raw bovine milk , out of which 384 from Dairy producers and 23 from Vendors were sampled for isolation and identifications of *Listeria* species by bacteriological examination, 85 (20.88%) proved positive for *Listeria*. The identification results showed that raw bulk milk from vendors were highly contaminated with *Listeria* species with the detection rates of 30.4% and raw milk from dairy producers were 20.3 %. Statistically there was no significant difference (P>0.05) with *Listeria* isolation among dairy producers and vendor at the study area (Table 8).

Table 8. Isolation and detections of *Listeria* species in raw milk from Dairy producers and Vendors.

Sources of raw milk sample	No. of sample examined	No. of Samples positive for *L. mono.* (%)	No. of Samples positive for other *Listeria* sps (%)	Total positive (%)	χ^2	P-value
Dairy p.	384	33 (8.59)	45 (11.7)	78 (20.3)	0.55	0.75
Vendors	23	3 (13.04)	4 (17.39)	7 (30.4)		
Total	**407**	**36 (8.8)**	**49 (12)**	**85 (20.88)**		

Dairy p. = Dairy producer; L. mono. = L. monocytogenes; sps= species

In this study, both pathogenic and non pathogenic *Listeria* species were isolated from raw milk samples of dairy producers and vendors in the study area. The detections of *L. monocytogenes* in both dairy producers and vendors were 8.6% and 13% respectively (Table 9).

Table 9. The prevalence of *L. monocytogenes* from different sources of samples

Source of sample	Total examined	Prevalence (%)	OR	95% CI of OR		P-value
				Lower	Upper	
Dairy producer	384	33(8.6)	REF*			
Vendor	23	3(13)	1.6	0.4503	- 5.6523	0.75
Total	407	36(8.8)				

OR= Odds Ratio; CI= 95% confidence interval, REF*= Reference.

As depicted in the above table (9), the level of identification of *L. monocytogenes* in vendor was 1.6 times (OR= 1.6, CI= 0.45-5.65) higher compared to milk samples originated from dairy producers. But it was not statistically significant (p = 0.75).

The bacteriological analysis indicated that *L. monocytogenes* and *L. innocau* were found with higher prevalence followed by *L. seeligeri.* The frequency of isolation of *Listeria* species from raw milks of the dairy producers and vendor are summarized as depicted below in (Table 10).

Table 10. Numbers of *Listeria* species isolated from raw milks of dairy producers and Vendors.

Listeria species Isolated	Dairy producers	Vendor	Total isolated spp.	Percentage (%)
L. monocytogenes	33	3	36	8.8
L. ivanovii	0	0	0	00
L. innocua	25	3	28	6.88
L. seeligeri	13	1	14	3.40
L. welshimeri	2	0	2	0.49
L. grayi	3	0	3	0.74
L. murrayi	2	0	2	0.49
Total *Listeria* isolates	**78**	**7**	**85**	**20.88**

4.2. Results of Antimicrobial susceptibility test

Of the total 36 *L. monocytogenes* species subjected for antimicrobial susceptibility test, 11(30.5%) exhibited resistance for Nalidixic acid, 9(25%), 8(22.2%) and 4(11.1%) of *L. monocytogenes* were resistance to Tetracycline, Chloramphenicol and Streptomycin's

respectively. All of *L. monocytogenes* isolated in this study were sensitive to Cephalotin, Kanamycin, Vancomycin and Ampcillin (Table 11) (Photo of antimicrobial susceptibility test shown in annex 9).

Table 11**.** Antimicrobial resistance profiles of *Listeria monocytogenes* from milk samples

Antimicrobial agent	Symbol	Disc Content	Numbers of isolates (%)		
			R	I	S
Cephalothin	KF	30 µg	-	-	36(100 %)
Chloramphenicol	C	30 µg	8(22.2 %)	10(27.7 %)	18(50 %)
Kanamycin	K	30 µg	-	-	36(100 %)
Nalidixic acid	NA	30 µg	11(30.5 %)	9(25 %)	16(44.4 %)
Streptomycin	S	10 µg	4(11.1 %)	5(13.8 %)	27(75 %)
Tetracycline	T	30 µg	9(25%)	5(13.8 %)	22(61.1 %)
Vancomycin	VA	30 µg	-	-	36(100 %)
Gentamicin	G	10 µg	-	7(19.4 %)	29(80.5 %)
Ampcillin	AMP	10 µg	-	-	36(100 %)

R= Resistance, I= Intermediate, S= sensitive

Of these L. monocytogenes exhibited resistance for one or more antimicrobials, one (2.7%) L. monocytogenes were resistances to four antimicrobials (Streptomycin, Chloramphenicol, Tetracycline and Naldixic acid). Nine (25%) L. monocytogenes were resistance to two antimicrobials. Ten (27.8%) of L. monocytogenes were resistance to one antimicrobial as indicated in Figure 5.

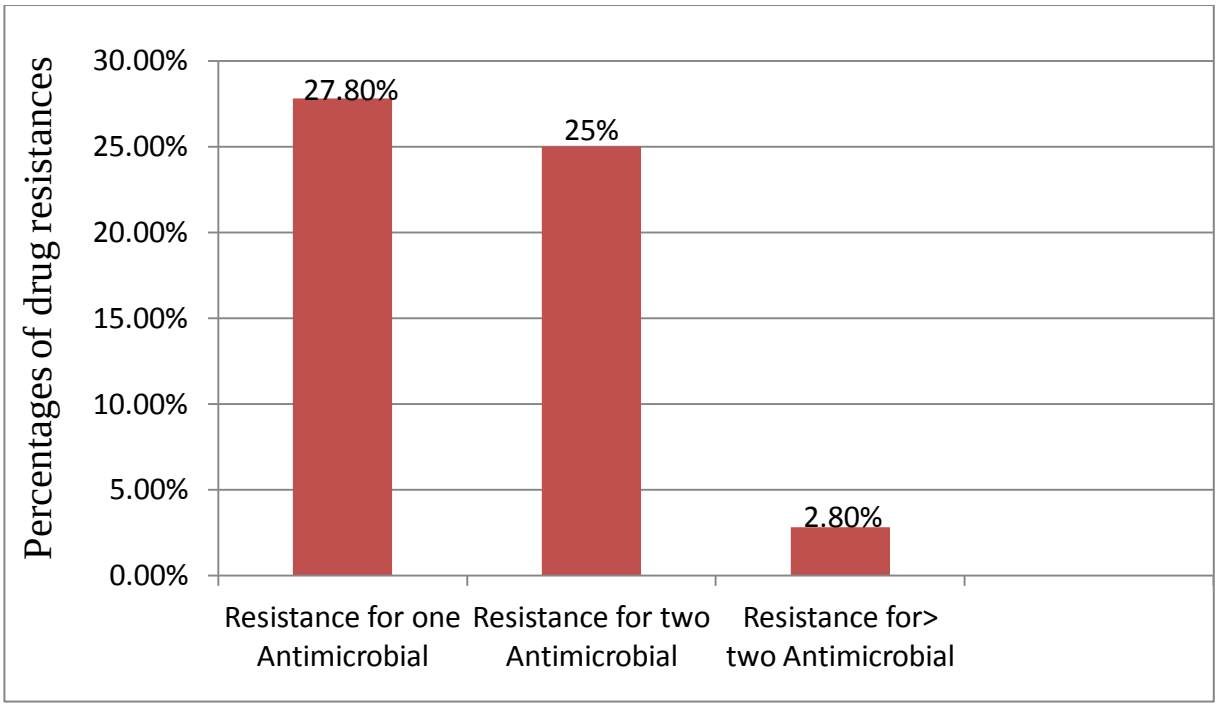

Figure 5. Resistance profiles of *Listeria monocytogenes* isolated from raw milk samples.

5. DISCUSSIONS

The present study showed that *L. monocytogenes* and other *Listeria* species were quietly detected in raw bovine milks from dairy producers and vendors at Debre-Birhan, Ethiopia. In this study raw milk samples were analyzed for the presence of *L. monocytogenes* and other *Listeria* species. The overall isolations of *Listeria* species were 20.88% from 407 raw milk samples examined.

In Ethiopia, there is no citable study conducted on the prevalence of *L. monocytogenes* and other species of *Listeria* in raw milk from dairy producers, but its occurrence was reported in various food products of animal origin (Molla *et al*., 2004 and Mengesha, 2005), although the prevalence's of *L. monocytogenes* in raw milks collected from different milk retailer shops, cafeterias, milk product shopping market and supermarkets were reported (Abera, 2007; Gebretsadik *et al.,* 2011; Muhammed *et al.*, 2013; Garedew *et al.,* 2015).

The present finding was comparable with other reports in Ethiopia; Addis Ababa 22 % from 100 raw milk samples and Gondar 25 % from 50 raw milk samples (Gebretsadik *et al.,* 2011; Garedew *et al.,* 2015) respectively. In others countries like Uganda, out of 40 samples of bulk raw milk tested, 60% were positive for *Listeria* species (Mugampoza *et al.,* 2011) which is with higher prevalence compared to the present studies(20.88%). Such differences are might be due to poor hygiene and sanitation activities in the milk production, processing and supplying chains, since the origins of contaminations of milk with *Listeria* are mainly faeces (Uhitil *et al.*, 2004). In Ethiopia relatively low prevalence's of *Listeria* were reported in Jimma town 14 % from 50 milk samples and in Addis Ababa 8.3% from 60 raw milk samples (Muhammed *et al.,* 2013; Abera, 2007) respectively. These different isolations might be due to either by the difference of samples sizes or hygienic practicing and /or sample collecting origin.

The isolated species of *Listeria* in the present study was *L. monocytogenes* 36(8.8%), *L.innoccau* 28(6.88%), *L. seeligeri* 14(3.4%), *L. grayi* (0.74%), *L. welshimeri* (0.49%) and *L. murrayi* (0.49%) indicated as diminishing orders.

In this study almost from both sampling origin the presence of *L. monocytogenes* in raw milk, higher bacterium isolation were observed in vendors. This might be due to supply chain contamination from dairy producer to the retailer transportations or might be the retailers' handling exposed to contaminations. Because of *L. monocytogenes* are found everywhere; in water, soil vegetables and in healthy persons without showing symptom (Liu, 2006). One of the main reasons for lower prevalence of *Listeria* in the dairy producers might be due to early supplying of the milk to collection centers where there are few chances of contamination from handlers and the environment (Abera, 2007).

Money researchers have been reported the occurrence of *Listeria* species in raw milks, according to Yakubu *et al.* (2012) report, out of 192 raw milk samples 76 (39.58%) were positive for *Listeria* species. Out of which 39 (20.3%) were *L. innocua,* 14 (7.3%) *L. ivanovii,* 17(8.9%) *L. monocytogenes,* 4(2.1%) *L. welshimeri* and 2(1%) *L. seeligeri.* Another studies conducted in Algeria by Hamdi *et al.* (2006) from raw milk samples, the isolations of *L. monocytogenes* were 7.7%. The detections of *L. monocytogenes* from raw milk was 6.5% in Ireland and 5.5% in Finland (Fox *et al.,* 2011; Ruusunen *et al.,* 2013) respectively. A total of 240 milk samples from farm bulk milk tanks collected in Tehran province, Iran, 54 (22.5%) were positive for *Listeria* species out of which *L. monocytogenes* 13(5.4%), *L. welshimeri* 6(2.5%) *L. innocau* 33 (13.8%) and *L. seeligeri* 2 (0.8%) (Hossein *et al.,* 2013). From these reports of *Listeria* species isolation, *L. monocytogene* were comparable with our findings.

Whereas other more recent reports in the isolations of *Listeria* species from unpasteurized raw milk were 13.46% and *L. monocytogenes* 4.81% (Saha *et al.,* 2015), relatively lower detection rates compared to the present studies. Similarly D'costa *et al.* (2012) study showed that the prevalence of *L. monocytogenes* was 4.82% in raw milk samples from different markets of Mumbai city, this study result was lower compared to the present findings. Ozkan and Bayacloglu (2004) reported 2% prevalence of *L. monocytogenes* in raw cow milks and Navratilova *et al.* (2004) reports in Czech, the prevalence of *L. monocytogenes* in raw bulk milk from dairy farm and raw milk from retailer tank were 2.1% and 5.1% respectively. These the above reports were lower isolation rates compared to the present studies.

On the other hand the antimicrobial profile of *L. monocytogenes* was also studied based on the standard Kirby Bauer technique, and it was done for all *L. monocytogenes* isolates. However, there is no standard interpretation chart for all types of drugs studied, for checking the breakpoint (cut off) value of the results for *L. monocytogenes*. Therefore, for most of the drugs; the results have been interpretated based on the standard breakpoints of NCCL's for Gram positive organisms, and only for Chloramphenicol and tetracycline, the actual zone of interpretation chart for *L. monocytogenes* was used (Rodas-Sua´rez *et al.*, 2006; Zhang *et al* ., 2007).

In the present finding the antimicrobial resistances of *L. monocytogenes* were 30.5%, 25%, 22.2% and 11.1% to Nalidixic acid, tetracycline, chloramphenicol and streptomycin respectively. Different studies revealed that the resistance of *L. monocytogenes* to streptomycin was 7.1% (Evrim *et al.*, 2012) and the resistance of *L. monocytogenes* to tetracycline has also been reported with 8.4% (Li *et al.*, 2007; Zhang *et al.*, 2007). Recent study conducted by saha *et al.* (2015) *L. monocytogenes* were 100% sensitivity to penicillin, tetracycline and gentamicin. The higher resistance of *L. monocytogenes* to tetracycline in the present study might be due to excessive use as it is relatively cheaper in price and easily accessible. Tetracycline is one of the most frequently prescribed drugs in the current study area for most of infectious disease in veterinary medicines. However, Jamali *et al.* (2013) had reported higher resistance of *L. monocytogenes* to tetracycline 34.6% compared to the present study. In agreement with the present finding, Morobe *et al.*(2009) were reported the resistances of *L. monocytogene* to tetracycline with 22.81% in Gaborone, Botswana.

All *L. monocytogene* isolated in the preset study were found to be intermediate and sensitive to Gentamicin. This finding is in agreement with studies undertaken by Abera (2007) in Addis Ababa, on the sensitivity of *L. monocytogene* to antimicrobials. Other reports in Ethiopia, on the susceptibility of *L. monocytogene* isolated from animal's faeces were 100% sensitive to Penicillin-G, Tetracycline, Ampicillin, Kanamycin, Vancomycin and chloramphenicols, following 96.3% Streptomycin and 92.6% Erythromycin (Wagari, 2006). This study results was inline with the present study in which *L. monocytogenes* was 100% sensitive to Ampicillin, Kanamycin and Vancomycin. Where as on tetracycline and chlorampnenicol, *L. monocytogenes* were showed resistance in the present study.

In the present study, majority of *L. monocytogenes* isolated were susceptible to Cephalotin and Vankomycin which was in agreement with the recent study in Gondar, Ethiopia (Garedew *et al.,* 2015). Moreover, reports by Rahimi *et al.*(2012) indicated that all isolated *L. monocytogenes* were 100% sensitive to Vancomycin. In the present study all *L. monocytogenes* isolated from raw milk were susceptible to Ampcillin which was agreed with the study conducted in Gaboron, Botswana (Morobe *et al.,* 2009).

Resistance of *L. monocytogenes* to nalidixic acid, tetracycline and chloramphenicol were observed in the present study which is similar with studies conducted by (Pintado *et al.,* 2005; Navratilova *et al.,* 2004; Sharma *et al.,* 2012). The presence of antimicrobial resistant *L. monocytogenes* in raw food products has an important public health implication especially in developing countries where there is a wide spread and uncontrolled use of antimicrobials (Sharma *et al.,* 2012). The problem can be higher in Ethiopia since consumption of raw meat, raw milk and raw milk products are very common and large number of high-risk population are found in the country.

The antimicrobial sensitivity of *L. monocytogenes* to Pencillin G(100%), tetracycline(100%), streptomycin(100%), cloxacillin and rifampicin, followed by chloramphenicol (81.5%), amoxicillin (81.5%) and vancomycin (81.5%), sulphamethazole/ trimethoprim (70.4%), ciprofloxacin (51.9%), gentamicin (48.2%), amikacin and neilmicin (40.7%) were reported in Egypt (Maha *et al.,* 2014). The increased antimicrobial administration to animals can cause the development of antimicrobial resistance *L. monocytogenes* (Schwartz *et al.,* 2003; Maha *et al.,* 2014). Multidrug resistant *L. monocytogene* isolated from foods and human Listeriosis has been previously reported by Safdar and Amstrong (2003) and Marian *et al*. (2012). The overall frequencies and patterns of resistance can vary remarkably from one country to another and also one reports to other even within a country.

6. CONCLUSIONS AND RECOMMENDATIONS

In this study the results of bacteriological assessment showed that milk from dairy producers and vendors are positive for *Listeria*. In this finding both pathogenic *L. monocytogenes* and non pathogenic *Listeria* species were isolated. The presence of *L. monocytogenes* in food can poses a threat to human life and can cause outbreaks of morbidity and mortality. Isolations of *L. monocytogenes* in raw milk and its antimicrobial resistant is an indication of public health hazards to the consumer, particularly to the high risk group shuch as young, elder, pregnant women and immune-compromised individuals. Based on the findings of the present study and the above conclusions the following recommendations are suggested:

- Raw milk intended for human consumption must be subjected for pasteurization or heat treatment at equivalent to pasteurization temperature.
- Further studies on the risk factors, molecular characterization and serotyping of the bacteria should be carried out.
- Awareness creation on food safety and implementation of regulations about the use of antimicrobials in humans and animals should be strongly practiced.

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8. ANNEXES

Annexs 1. Laboratory Data collection sheet

S. N	Sample code	Isolation and primary identification			Confirmation				
		Gram stain	Catalase test	Motility test	Hemolysis test	CAMP test	Carbohydrate utilization test		
							Mannitol	Rhamnose	Xylose

Annexs 2: Procedure of Gram's staining

1. Prepare the smear and fix with heat.
2. Stain with crystal violet for 60 seconds and rinse with tap water and drain off excess water.
3. Cover the smear with Gram's iodine solution and let it for 60 seconds and rinsing with tap water and drain.
4. Hold the slide at 45 degree angle and allow 95% ethanol until 15 seconds and rinse with tap water and drain.
5. Counter stain with safranin for 60 seconds and rinse with tap water and drain.
6. Examine the slide under oil immersion.

Interpretation: up on examining the slides the bacterial cell were differentiated by stain color Gram's positive bacteria have a dark blue to purple stain colors, while Gram's negative bacteria have a pink to red color. In this study, the target bacteria which is *Listeria* showed a

dark blue to purple stain color with slim and short rod cell morphology meaning that it is a Gram positive bacteria.

Annexs 3: Procedure for catalase test

1. Place a drop of 3% H_2O_2 on a glass slide.
2. Touch a sterile loop to a culture of the organism to be tested and pick up a visible mass of cells (colony).
3. Mix the organism in the drop of hydrogen peroxide.
4. Observe for immediate and vigorous bubbling.

Interpretation: Bubbling indicates a positive test and no bubbling indicates a negative test.

Annexs 4: Procedure for haemolysis test

1. Isolates colony was taken with an inoculating needle from a typical colony from TSA.
2. Streak the sample in to 5% Sheep Blood Agar Base.
3. It was incubated at 37°C for 24 hours.
4. After incubation positive test cultures show narrow, clear and light zones (β-haemolysis).

Annexs 5: Procedure for CAMP test

1. Take a colony culture with an inoculating needle from a typical colony on TSA
2. Staphylococcus aureus was taken (ATCC 25925).
3. It was streaked vertically in a single line across a sheep blood agar plate and *Listeria* isolates horizontally to S. aureus streak and
4. The plates were incubated at 37^{o}C for 18 to 24 hours.
5. An enhanced zone of beta hemolysis between the test strain and culture of S. aureus was considered a positive reaction. *L.monocytogenes* showed an enhanced zone of hemolysis, forming anarrow head towards the S. aureus culture.

Annexs 6: Procedure for carbohydrate utilization test

1. Isolated colonies from TSA was transferred into test tubes containing xylose, rhamnose and mannitol and
2. It was incubated at 37^{o}C for up to 5 days.
3. Positive reactions were indicated by yellow color (acid formation).

Annexs 7: Composition and Preparation of culture media and broth used for the study

1. Half Fraser Broth Base (HIMEDIA, INDIA, MUMBAI)

Specifications; M1327

Use; Fraser Broth Base is enrichment medium, used for the isolation and enumeration of *Listeria monocytogenes* from food and animal feeds.

Composition:

Ingredients	Gms/lit
Peptic digest of animal tissue	5.000
Casein enzymic hydrolysate	5.000
Yeast extract	5.000
Meat extracts	5.000
Sodium chloride	20.000
Disodium hydrogen phosphate.$2H_2O$	12.000
Potassium dihydrogen phosphate	1.350
Esculin	1.000
Lithium chloride	3.000

Final pH (at 25°C) 7.2±0.2

Directions;

Suspend 54.92 grams (equivalent weight of dehydrated medium per liter) in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add rehydrated contents of 1 vial of Fraser Selective Supplement (FD125I) and 2 vials of Fraser Supplement (FD141) to 1000 ml medium.

2**.** Fraser Broth Base (HIMEDIA, INDIA, MUMBAI)

Specifications; M1327

Use; Fraser Broth Base is enrichment medium, used for the isolation and enumeration of *Listeria monocytogenes* from food and animal feeds.

Composition:

Ingredients	Gms/lit
Peptic digest of animal tissue	5.000

Casein enzymic hydrolysate	5.000
Yeast extract	5.000
Meat extracts	5.000
Sodium chloride	20.000
Disodium hydrogen phosphate.$2H_2O$	12.000
Potassium dihydrogen phosphate	1.350
Esculin	1.000
Lithium chloride	3.000

Final pH (at 25°C) 7.2±0.2

Directions;

Suspend 27.46 grams (equivalent weight of dehydrated medium per liter) in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add rehydrated contents of 1 vial of Fraser Selective Supplement (FD125I) and 1 vials of Fraser Supplement (FD141) to 500 ml enrichment. Mix well and dispense to test tubes.

Fraser Selective Supplement

Specifications (FD125I)

Recommended for selective isolation and enumeration of *Listeria monocytogenes* from food, animal feeds etc.

Ingredients	Concentrations
Acriflavin hydrochloride	12.500mg
Nalidixic acid	10mg

Directions:

Rehydrate the contents of 1 vial aseptically with 10 ml of sterile distilled water. Mix well.

Fraser Supplement

Specifications (FD141**)**

Recommended by ISO Committee for the selective isolation and enumeration of *Listeria monocytogenes* from food, animal feeds etc.

Ingredients	concentrations

Ferric ammonium citrate	250mg

Directions:

Rehydrate the contents of 1 vial aseptically with 2 ml of sterile distilled water. Mix well.

3. *LISTERIA* SELECTIVE AGAR

Listeria Oxford Medium Base **(**HIMEDIA**,** INDIA, MUMBAI)

Specifications (M1145)

Use: A selective medium for the isolation of *Listeria monocytogenes* from food and clinical materials.

Ingredients	Gms/ Litre
Peptone, special	23.000
Lithium chloride	15.000
Sodium chloride	5.000
Corn starch	1.000
Esculin	1.000
Ammonium ferric citrate	0.500
Agar	10.000
Final pH (at 25°C) 7.0±0.2	

Directions

Suspend 27.75 grams in 500 ml distilled water. Heat to boiling to dissolves the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add the rehydrated contents of 1 vial of Oxford *Listeria* Supplement (FD071). Mix well before pouring into sterile Petri plates.

Oxford *Listeria* Supplement

Specifications (FD071)

An antimicrobial supplement recommended for selective isolation of *Listeria* species.

Composition:

Ingredients	Concentration
Cycloheximide	200mg
Colistin sulphate	10mg

Acriflavin	2.500mg
Cephazolin	1mg
Fosfomycin	5mg

Directions:

Rehydrate the contents of 1 vial aseptically with 5 ml of 50% ethanol. Mix gently to dissolve the supplements.

4. Tryptone Soya Yeast Extrac Agar (SIGMA-ALDRICH, St. Louis, USA)

Specifications (93395)

Use: Tryptone soya yeast extract agar is recommended for conformation of *Listeria* and used for cultivation of a wide variety of microorganisms and for sterility testing.

Composition

Ingredients	Gms/Liter
Casein enzymic hydrolysate	17.0
Papaic digest of soyabean meal	3.0
Dipotassium hydrogen phosphate	2.5
Dextrose	2.5
Sodium chloride	5.0
Agar	15.0
Yeast extract	6.0
Final pH (at 25°C)	7.3±0.2

Directions: Suspend 51grams in 1000ml distelled water. Heat to boiling to dissolve medium completely. Sterilize by autoclaving at 15 lbs pressure 121°C for 15 minute. Mix well and pour into sterile perti plates.

5. Tryptone soya yeast extract broth (SIGMA-ALDRICH, St. Louis, USA)

Specifications (55309)

Use: Tryptone soya yeast extrac broth is recommended for confirmation of Listeria.

Composition

Ingredients	Gms / Litre
Casein enzymic hydrolysate	17.0
Papaic digest of soya bean meal	3.0

Dipotassium phosphate	2.5
Glucose	2.5
Sodium chloride	5.0
Yeast extract	6.0
Final pH (at 25°C)	7.3±0.2

Directions: Suspend 36 grams of in 1000ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure 121°C for 15 mnutes. Mix well and dispense as desired on strile test tubes.

6. Blood agar(Becton Dickinson, Maryland, USA)

Composition	gm/litre
Heart infusion from (solids)	2.0
Pancreatic digest of casein	13.0
Yeast extract	5
Agar	15.0
Sodium chloride	5.0

Directions: Suspend 40gm powder dissolved in 1liter of distilled water and mix well.

Heat with frequent agitation and boil for one minute to completely dissolve the powder.

Autocalve at 121°C for15minutes. Cool the base to 45 to 50oC and add 5% sterile, defibrinated sheep blood. Dispense in to petridishes.

7. Mueller hinton agar (SIGMA-ALDRICH, St. Louis, USA)

Specifications; 70191M-H agar.

Use: A solid medium widely used for antibiotic susceptibility testing

Composition	Grams/litre
Beef infusion solids	2.0
Starch	1.5
Casein hydrolysate	17.5
Agar	17.0
Final pH 7.3 +/- 0.2 at 25°C.	

Directions: Suspend 38gm in 1litre of distilled water. Mix thoroughly, heat with frequent agitation and boil for 1 minute to completely dissolve. Sterilize by autoclaving at 121°C for 15 minutes.

8. Mueller hinton broth (SIGMA-ALDRICH, St. Louis, USA)

Use: A liquid medium for antibiotic susceptibility studies

Specification: 70192 M-H Broth

Composition:

Ingredients	Grams/Litre
Beef infusion solids	2.0
Starch	1.5
Casein hydrolysate	17.5

Final pH 7.4 +/- 0.2 at 25°C

9. Carbohydrate utilization broth (rhamnose, xylose and mannitol)

i. Phenol red

Composition	gm/l
Peptone from meat	5
Peptone from casein	5
Sodium chloride	5
Phenol red	0.018

Direction: Dissolve 15gm of powder in 1lit of purified water. Autoclave at 121°C for 15 minutes and cool to about 60°C

ii. Carbohydrate solutions

Rhamnose, Xylose and Mannitol

Directions: Dissolve 5gm of each carbohydrate in 100ml of water separately Sterilization by filtration

iii. Complete medium

Direction: For each carbohydrate, add aseptically 0.5ml of filter sterilized carbohydrate solution to 4.5ml of phenol red solution prepared.

Annexs 8: Zone Diameter Interpretive Standards (Kirby Bauer disk diffusion method NCCLS, 2004)

Antimicrobial agent	Symbols	Disc Content (µg)	Zone Diameter (mm)			
			S	**I**	**R**	Comments
Cephalothin	KF	30	≥18	15-17	≤14	G.P
Chloramphenicol	C	30	≥21 ≥ 18	18-20 13-17	≤ 18 ≤ 12	L.m * G.P
Kanamycin	K	30	≥ 18	14-17	≤ 13	G.P
Nalidixic acid	NA	30	≥ 19	14-18	≤ 13	G.P
Streptomycin	S	10	≥ 15	12-14	≤ 11	G.P
Tetracycline	T	30	≥ 25 ≥19	22-24 15-18	≤ 22 ≤ 14	L.m* G.P
Vancomycin	VA	30	≥ 12	10-11	≤ 9	G.P.
Gentamicin	G	10	≥ 15	13-14	≤ 12	G.P.
Ampcillin	AMP	10	≥ 14	12-13	≤ 11	G.P

Keys; G.P = Gram positive, *L.m = *Listeria monocytogenes*

S = Sensitive, I = Intermediate, R = Resistance

Annexs 9. Photographs of Antimicrobial growth inhibition zone on antimicrobial susceptibility test