Detection of Possible Sources of *Listeria monocytogenes* and other *Listeria* species and their Antimicrobial Resistance Profiles along Haramaya University Dairy Farm Operational stages, Oromia Regional State, Ethiopia

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DEDICATION

I dedicated this Thesis to my mother Shito Aliyi, my father Mohammed Ahmed, my sole mate wife, Roza Sabit and my Son Faiz Hussein because without their patience and support I wouldn't have been able to achieve so much.

STATEMENT OF THE AUTHOR

By my signature below, I declare and affirm that this thesis is my own work. I followed all ethical and technical principles of the scholarship in the preparation, data collection, data analysis and completion of this thesis. Any scholarly matter that is included in the Thesis has been given recognition through citation.

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BIOGRAPHICAL SKETCH

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ACRONYMS AND ABBREVIATIONS

ALOA	Agar *Listeria* according to Ottaviani and Agosti
BLEB	Buffered *Listeria* Enrichment Broth
BPW	Buffered Peptone Water
CAMP	Christie–Atkins–Munch–Peterson
CDC	Center for Disease Control
CFU	Colony Forming Units
DALYS	Disability-Adjusted Life Years
FAO	Food and Agricultural Organization
FDA	Federal Drug Administration
FSIS	Food Safety and Inspection Service
ISO	International Standard Organization
LPM	Lithium chloride/phenylethanol/moxalactam
MDR	Multi Drug Resistance
MOX	Modified Oxford agar
NCCLS	National Committee for Clinical Laboratory Standards
OCLA	Oxoid Chromogenic *Listeria* Agar
ONE	Oxoid Novel Enrichment
PALCAM	Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol
PCR	Polymerase Chain Reaction
RTE	Ready-To-Eat
TSB YE	Trypticase–Soy Broth containing Yeast Extract
USDA	United States Department of Agriculture
WHO	World Health Organization
YOPI	Young, Old, Pregnant, and Immune-compromised

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ABSTRACT

Listeriosis is one of the most important emerging bacterial infections worldwide that arises mainly from the consumption of contaminated food including raw milk. A cross-sectional study was conducted from December 2017 to May 2018 in Haramaya University Dairy Farm. The objective of this study was to detect the possible sources of *L. monocytogenes* and other *Listeria* species along Haramaya University Dairy Farm's operational stages and to assess the antimicrobial resistance profiles on the isolates. Purposive type of study with random sampling method was used to select six dairy operational stages and fourteen sampling locations within the farm. A standard culture reference method recommended by ISO 11290 Protocols was applied for laboratory procedures. The isolates were tested for resistance against selected antimicrobials using disk diffusion technique. Out of 200 samples, 40 (20%) were positive for overall *Listeria* species from all operational stages and the majority of sampling locations were also positive. The distribution of *Listeria* species at operational stages were 30%, 26.7%, 20%, 18.6% and 10% at cow barn, milk supply, silage and milk from cow teat, milking operation and milk auditing respectively. *L. monocytogenes* was detected in 11(5.5%) of all samples with 2.8%, 6.6%, 7.5% and 10% from milking operation, milk supply station, feed and watering and cow barn and milk from cow teat respectively. The frequency of other *Listeria* species were, *L. innocua* (5%), *L. gray* (3%), *L. ivanovii* (2.5%), *L. seeligeri* (2%) and *L. welshimeri* (2%) in descending order. The tested isolates were found resistant at varied frequency to tetracycline (55%), penicillin (45%) and amoxicillin (20%) were observed. Likewise 54.5% of *L. monocytogenes* showed resistance to tetracycline and equal (27.3%) isolates were resistance to each of amoxicillin and streptomycin. Of the total isolates, 82.5% shows single to multi drug resistance in which 14 (35%), 10 (25%), 7(17.5%) and 2(5%) isolates were resistance to single, two, three and four drugs respectively. The detected *L. monocytogenes* and other *Listeria* species from majority of sampling stages and sampling locations can act as sources of milk contamination that becomes a public health risk. Therefore, hygienic practices should be implemented at all stages and locations for prevention and control of *Listeria* and the resistant isolates.

Keywords: Antimicrobial resistance, *Dairy farms operational stages, Detection, L.monocytogenes, Listeria spp, Silage*

1. INTRODUCTION

Listeriosis is one of the most important emerging bacterial infections worldwide that arises mainly from the consumption of contaminated food (Kalorey *et al*., 2008). The genus *Listeria* comprises seven species including *L. monocytogenes* (*Listeria monocytogenes), L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. murrayi and L. grayi*. Among the different species *L. monocytogenes* is pathogenic which is considered an opportunistic agent that affects mainly those with underlying immune conditions, such as pregnant women, neonates, and elders, resulting in septicemia, meningitis, and/or meningo-encephalitis (Posfay and Wald, 2004).

L. monocytogenes is a Gram-positive bacterial pathogen that can cause foodborne illness ranging from mild illness (listerial gastroenteritis) to severe and often fatal illness in case of invasive listeriosis (Painter and Slutsker, 2007) both in humans and animals (Briandet *et al.*, 1999). Although listeriosis is relatively rare, the mortality rate reaches 25% worldwide as food borne microbial pathogen(de Noordhout *et al*., 2014) and its infections are responsible for high hospitalization from which 91% among the most common food-borne pathogens (Jemmi and Stephan, 2006).

Various types of food were deemed to be potentially contaminated with this pathogen and found widely in water, soil, infected animals, human and animal feces, raw and treated sewage, leafy vegetables, effluent from poultry and meat processing facilities, decaying corn and soybeans, improperly fermented silage, raw (unpasteurized) milk. Recent reports indicated that *Listeria* species including *L. monocytogenes* is most frequently prevalent in the milk-processing environment including steps, drains and floors (Kells and Gilmour, 2004).

However, despite advances in food preservation technologies advancement in diagnosis and laboratory techniques, *L. monocytogenes* continues to be public health problem concerned with the control of this bacterium, particularly with respect to the contamination of food (McLauchlin *et al*., 2004). Several European countries indicates increasing rate of Listeriosis (Allerberger and

Wagner, 2010), and occurrences of outbreaks in the United States (Cartwright *et al*., 2013), Canada (Taillefer *et al.*, 2010) and China (Wang *et al*., 2013).

In Ethiopia, there are detection and isolation of the *L. monocytogenes* and other *Listeria* species were reported from foods, at Addis Ababa (Bayeleyegn *et al*., 2004; Simon *et al*., 2011; and Firehiwot *et al*., 2013), Gondar (Legese *et al*., 2015), central highlands of Ethiopia (Eyasu *et al*., 2015), Bishoftu and Dukem (Sintayehu, 2017).

Although foods of animal origin including milk and milk products are consumed well in Ethiopia, published information on the status of food borne listeriosis caused by *L. monocytogenes* is very limited both in the Veterinary Medicine and Public Health sectors in Eastern part of the country. Even though those few studies reported *L. monocytogenes* detected in foods of animal origin including milk, there is no any structured study indicating detection of this pathogen from dairy farm environments and silage feed supplied to dairy cows as possible source of milk contamination in Ethiopia. It is also helpful for future research work designed in large scale as well as for surveillance of food-borne diseases caused by these pathogens in the Haramaya University and as well in Eastern Ethiopian community.

Thus, the objectives of this study were:

- To detect the possible sources and occurrence of *L. monocytogenes* and other *Listeria* species along Haramaya University Dairy Farms' operational stages.
- To assess antimicrobial resistance profiles of *L. monocytogenes* and other *Listeria* species isolated from dairy farms' operational stages.

2. LITERATURE REVIEW

2.1. Taxonomic Classification of Genus *Listeria*

Bacteria of the genus *Listeria* are Gram-positive, facultative anaerobic, non-spore forming rods and all members of the genus *Listeria* are widely distributed in nature and have been isolated from soil, vegetation, sewage, water, and animal feed, fresh and frozen meat including poultry, slaughter house wastes and in the feaces of healthy animals including humans(Simon *et al*.,2010). These organisms can become endemic in food processing environments. The genus Listeria includes seven species: *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L.* i*vanovii, L. murrayi, L. grayi* and four subspecies. Four species have been implicated in cases of human illness, but *L. monocytogenes* is by far the most important and is the cause of most reported cases of human infection. The species is made up of at least 13 different serotypes, all of which can cause listeriosis, but the most commonly found in human infection is serotype 4b (Jeyaletchumi *et al*., 2010).

Listeria monocytogenes is ubiquitous in the environment and can be isolated from soil, plant material, animals and even marine fish and seafood. It has also been found in a very wide variety of food products, but its presence is particularly hazardous in chilled processed foods with a long shelf life, such as smoked fish, pâtés, soft cheeses and ready-to-eat cooked meats. This is because it is able to grow slowly at temperatures as low as 0^0C and may therefore multiply to dangerous levels in refrigerated foods unless controlled. *Listeria* species are also common colonizers of food factories where they may form biofilms that are difficult to remove. These biofilms can act as reservoirs of persistent *L. monocytogenes* contamination for processed foods if not controlled. The presence of *Listeria* species in the food factory environment is often used as an indicator for *L. monocytogenes* contamination. Elimination of *Listeria* from many environmental sources can be challenging because of the resistant, persistent biofilms formed by these pathogens (Doyle, 2013).

Although *L. monocytogenes* is widely distributed in the environment, outbreaks of listeriosis tend to be seasonal in European countries and to affect silage-fed animals in late pregnancy. *Listeria*

monocytogenes can replicate in the surface layers of poor-quality silage with pH values above 5.5. In such circumstances, listerial numbers may reach $l0^7$ colony-forming units per kg of silage. In good quality silage, multiplication of the organisms is inhibited by the acid produced by fermentation. Susceptibility to infection with *L. monocytogenes* has been attributed to decreased cell-mediated immunity associated with advanced pregnancy (Quinn *et al*., 2008).

Listeria monocytogenes is an important food-borne pathogen not because it causes large numbers of symptomatic cases but because of its relatively high case–fatality rate. In listeriosis cases, most victims are elderly, immune-compromised, or pregnant and *Listeria* is invasive, causing bacteremia, meningitis, or illness or death in a fetus or newborn infant with median incubation periods are 2, 9, and 27.5 days, respectively are particularly at risk (Doyle, 2013).

As described by Doyle (2013) Listeriosis appears to be primarily a food borne infection and is particularly a problem on foods that are not cooked just prior to consumption, including ready-to-eat (RTE) meats, soft cheeses, and unpasteurized dairy products, as well as sprouts, salad vegetables, and fruit. Thermal processing of milk and meat will destroy *L. monocytogenes* but post-processing contamination does occur. Because this pathogen grows during refrigeration, simply keeping foods cold does not ensure their safety.

About 94% of listeriosis cases are hospitalized and about 16% die. Despite the widespread occurrence of *L. monocytogenes* in the environment, relatively few exposed people become ill. Some outbreaks of listeriosis resemble other food borne illness, with symptoms of gastro-enteritis and fever occurring after a median incubation period of 24 hours. *Listeria* concentrations in implicated food may be quite high (10^4–10^9cfu/g), and cases often do not have well-established risk factors (Doyle, 2013).

2.2. Growth Characteristics of *Listeria monocytogenes*

Listeria monocytogenes, unlike other pathogens*,* can grow at refrigeration temperatures and in foods with fairly low moisture content, high salt concentration and adapt to changes in acidity. Factors that make *L. monocytogenes* especially difficult to control are its ability to resists ordinary disinfectants and survives exposure to various stresses and food preservation methods such as low pH, low temperatures and the presence of salt, thus, enabling the pathogen to survive

on surfaces in food processing facilities, in the human gut, during refrigeration and in marine waters. Under stressful conditions *L. monocytogenes* can form long filaments, which may result in an underestimation of risk in certain foods (Doyle, 2013).

Listeria species can grow over a wide range of temperatures (- 1.5 to 45-50°C) and pH ranges (4.3-9.6), survives freezing, and is relatively resistant to heat. Minimal water activity levels for growth of *L. monocytogenes* and *L. ivanovii* range from 0.90 to 0.97. *L. monocytogenes* also reported survival for 132 days at 4°C in Trypticase Soy Broth containing 25.5% NaCl. *Listeia*is a psychrotrophic pathogen and growth at temperatures as low as -0.1 to -0.4^{0}C in chicken broth and pasteurized milk and at - 1.5^{0}C in vacuum-packaged meat has been recorded (Vail *et al.,* 2012; Raheem, 2016).

As reported by Doyle (2013), a risk assessment tool has been developed to aid in determining risk for significant *Listeria* levels in cold foods that are transported without adequate temperature control. Numerous investigations, over many years, have recorded data on growth and survival of *Listeria* species in a variety of foods. *Listeria* is known to survive for extended periods in acidic conditions and can form biofilms to grow in the absence of oxygen. In recent years, biofilms have been shown to be important for harborage of various food-borne pathogens thereby increasing the risk for food safety violations. Contamination of food products are fostered by biofilms on equipments and their formation is favored by the accumulation of food residues, spoilage organisms and food-borne pathogens. Many food manufacturing processes may provide niches of low sanitation level, where biofilms can prosper, including stainless steel (ultra filtration membrane, valves, and air separator), rubber surfaces (belt, packing machine, air separator, liner, short milking tube) and floors (Raheem, 2016).

2.3. Sources of Milk Contamination with *Listeria monocytogenes* and other *Listeria* species

Listeria is widely present in the environment, and there are many potential pathways by which food may be contaminated. The main factors to be considered in food contamination are; the initial source of the bacteria, the ability of the food to support the bacteria and post-processing contamination of food. The most common sources of *L. monocytogenes* include raw and processed meat, dairy products, vegetables and seafood products. Also, food production facilities and storage environments have importance as sources. Refrigerated foods that may be consumed without prior cooking are of special concern because of the ability of *L. monocytogenes* to grow at refrigeration temperatures (Leong *et al*., 2014).

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, 2013). Employees handling food may also spread *Listeria* and facilitate cross-contamination in production facilities and food preparation areas (Doyle, 2013).

Listeria monocytogenes is also present in feces of Cattle, sheep and goats and may be secreted into milk if a cow or goat or ewe has mastitis. A trace-back investigation of contamination of raw ewe's-milk cheese in Austria found that ewes with mastitis were shedding an average of 3×10^4cfu *L. monocytogenes*/ml of milk (Doyle, 2013) and mastitis caused by *Listeria* is infrequent, but infected mammary glands can shed this pathogen for periods as long as 12 months (Winter *et al*., 2004). Recent investigations at a dairy farm indicating that a positive tests for *L. monocytogenes* in bulk tank milk revealed the presence of this bacterium in 66% of milk filters, 16% of bulk tank milk samples, and 6% of water samples. It appeared that there was a common source of contamination and the most likely sources were believed to be an asymptomatic cow shedding *L. monocytogenes* or some site within the milking machine where *L. monocytogenes* could survive and grow (Pantoja *et al.,* 2012).

The populations of *L. monocytogenes* have been shown to survive minimum pasteurization treatments of 71.1 ^{0}C/ 16sec in various laboratory studies, survival under actual conditions of commercial milk pasteurization and processing is un-likely. *Listeria* contamination of processed dairy products is most likely a function of post pasteurization from the dairy plant environment, and numerous surveys documented presence of *Listeria* within the dairy plant environment (Pantoja *et al.,* 2012; Donnelly, 1999).

Sources of *Listeria* within the dairy plant environment include floors in coolers, freezers, and processing rooms, particularly entrances; cases and case washers; floor mats and foot baths; and the beds of paper fillers. It is also in a study of dairy-processing facilities; found that those processing plants having a farm contiguous to the processing facilities had a significantly higher incidence of *Listeria* contamination than farms without an on-site dairy farm. Another study used ribo type analysis to demonstrate the link between on-farm sources of *Listeria* contamination (dairy cattle, raw milk, and silage) and subsequent contamination of dairy-processing environments. Raw milk is a well-recognized source of *Listeria,* and for this and numerous other microbiological reasons, consumption of raw milk should be avoided (Donnelly, 1999).

2.4. Public Health Importance of *Listeria*

Listeria monocytogenes is a food-borne bacterial pathogen that can cause listeriosis, a severe disease that can result in septiceamia, meningitis and spontaneous abortion (FAO and WHO. 2006). Although *L. monocytogenes* is infective to all human population groups, it has a propensity to cause especially severe problems in pregnant women, neonates, the elderly and immune-suppressed individuals (Abdi *et al*., 2016).

Direct transmission is possible especially among veterinarians performing gynecological interventions with aborted animals. Animals may be diseased or asymptomatic carriers of *L. monocytogenes* shedding the organism in their feces. Thus, earlier it was believed that *L. monocytogenes* was causing disease by direct transmission from animals to humans.

Abdi *et al.,* (2016) reported that indirect transmission may occur simply by consumption of food products from diseased animals on-farm manufactured raw milk cheese made from cattle with subclinical infection caused an outbreak with febrile gastrointestinal listeriosis involving 120 people. Raw or contaminated milk, vegetables and ready-to-eat meat have been implicated in overseas outbreaks. Contamination could be during preparation and it then multiplies during the storage process. Unlike some other food-borne pathogens, *Listeria monocytogenes* can multiply in contaminated refrigerated food (Heymann, 2004). For example, soft cheese is considered as a high risk product for listeriosis because the bacteria may grow to significant numbers during refrigeration. An outbreak was involved consumption of contaminated unpasteurized milk and other dairy products such as cheese and yoghurt. Transmission of *L. monocytogenes* from mother to foetus or neonates has been frequently reported but cross-infection postpartum is also possible (Abdi *et al*., 2016)

In neonatal infection, the pathogen is transmitted from the infected mother to her fetus trans-placental following maternal bacteremia. Some infections may also occur through ascending spread from vaginal colonization with the fetus acquiring infection during passage through the birth canal. Other routes of transmission, e.g. outbreaks attributed to contaminated equipment or materials in delivery room and hospital have also been reported (Abdi *et al.*, 2016).

If normal healthy adults acquire infection, the disease usually presents as a mild febrile illness resembling influenza. Papular lesions on the hands and arms, principally in veterinarians and farmers, can result from contact with infective material. Infection with *L. monocytogenes* can lead to abortion in pregnant women and can be life-threatening in neonates, the elderly and in immuno- suppressed individuals. Direct transfer from infected animals to humans is uncommon and is of little consequence in healthy, non-pregnant individuals (Vazquez-Boland *et al*., 2001).

Listeria monocytogenes causes both perinatal and adult listeriosis in humans. Perinatal human listeriosis may cause intrauterine infection resulting in intrauterine sepsis and death before or after delivery. Adults can develop meningo-encephalitis, bactermia and sometimes focal infections (Vazquez-Boland *et al*., 2001). While 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 immune-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 (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; the increased proportion of susceptible people due to age, immune-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 and others, especially which are known to harbor dangerous pathogens (Oliver *et al.*, 2005).

2.5. Global *Listeria* Outbreaks Reports

Diseases caused by food borne pathogens constitute a worldwide public health problem. Listeriosis; a food borne disease, has been considered to be an emerging zoonotic disease worldwide; which cause dangerous illness most commonly by *Listeria monocytogenes.*

Listeriosis outbreaks linked to consumption of unpasteurized or cross contaminated dairy products have occurred (CSPI, 2008). The high fatality rate, occurrence of outbreaks, and ability of *L. monocytogenes* to contaminate both unpasteurized and pasteurized foods has led the Microbiological specifications for food items including milk and dairy products often stipulate absence of *L. monocytogenes* (Roberts *et al.*, 1995)

According to Raheem (2016) *L. monocytogenes* was estimated to infect 23, 150 people globally and killed 5,463 (23.6%) of the infected people in 2010. Recently from selected reports of *Listeria* outbreaks globally from 1995 to 2016 by Raheem (2016) indicated in 1995 in France 36 cases due to Cheese; in 1997 in Sweden 9 cases due to Rainbow trout; from 1998–1999 in USA 108 cases by Frankfurter; in 2002 in Canada 130 cases due to Soft cheese; in 2006/7 in Germany

189 cases due to Jellied pork; from 2008–2013 in Netherlands 406 outbreak cases; in 2012 in South Africa 51 cases due to RTE foods; in 2016 in USA 19 cases by Lettuce salad have been reported. However, most of the cases involving *Listeria* are not reported in developing countries due to ineffective control or surveillance strategies. This makes it problematic in having a comprehensive global outlook on the outbreaks of *Listeria* and listeriosis in humans. These cases were either from the raw food samples, the processing plants of such foods or the processed foods in different countries (Raheem 2016).

As trace back reports of CDC since 1970 analyzed that, *L. monocytogenes*is the third most costly foodborne pathogen in the USA per case in 2010, after *Clostridium botulinum* and *Vibrio vulnificus*. The global burden of human listeriosis was estimated for 2010 to result in 172, 823 disability-adjusted life years (DALYs). DALYs are useful in comparing diseases and health conditions thereby it will helppolicy makers to allocate resources in the prevention of listeriosis. It was estimated that the annual benefit from safety measures of *L. monocytogenes* in the USA far exceeds its cost. The estimated benefit and cost was US$ 2.3 billion to 22 billion, and US$ 0.01 billion to 2.4 billion respectively (Raheem, 2016).

Although the disease burden of listeriosis on population level is low, on individual level the impact is high, largely due to severe illness and a high case fatality. Despite this low incidence of listeriosis that represents less than 0.1% of all food-borne illnesses they cause infections with very high mortalities 20 to 30% deaths (Raheem, 2016).

2.6. Reports of *L. monocytogenes* status from Ethiopia

An overall prevalence of *Listeria* species: 14% (Muhammed *et al*., 2013) at Jimma to 32.9% (Sintayehu, 2017) were reported. Regarding *L. monocytogenes*, 3.8% (Selamawit, 2014) to 6.25% (Legese *et al*., 2015) were reported in foods of animal origins. As in other developing countries, in Ethiopia, there are no well-organized epidemiological surveillance systems and few studies in limited areas so far are summarized in the Table 1 below.

Table 1. Overall *Listeria* and *L. monocytogenes* reports in food items sampled from Ethiopia

Studied area	Food item sampled*	Overall *Listeria* Spp. prevalence (%)	*L. monocytogenes* prevalence (%)	References
Addis Ababa	Retail meat and milk products	32.6	5.1	Bayeleyegn *et al*., 2004
Not specified	RTE foods and raw meat products	26.6	4.8	Mengesha *et al*., 2009
Addis Ababa	Foods of animal origin	26.1	5.4	Simon *et al.,* 2011
Addis Ababa and its surrounding town	Retail meat and dairy products	27.4	5.4	Firehiwot *et al*., 2013
Addis Ababa	Sheep meat from abattoir and butcher shops	-	3.8	Selamawit, 2014
North Shewa	Raw milk and dairy products	24.2	4.4	Tefera, 2014
Central Highlands of Ethiopia	Raw milk and milk products	28.4	5.6	Eyasu *et al*., 2015
Gondar town	RTE foods of animal origin	25	6.25	Legese *et al*., 2015
Bishoftu and Dukem	Foods of animal origin	32.9	5.8	Sintayehu, 2017
Debre-Birhan Town	Raw bovine milk	20.88	8.84	Yeshibelay and Abebe, 2018

*RTE = Ready to Eat-Food

In Ethiopia, *L. monocytogenes* were reported in various foods of animal origin (1 – 69.8%) of different levels. In dairy products, 1% in Cottage cheeses (Simon *et al*., 2011) to 43.5%% in ice cream (Bayeleyegn *et al*., 2004) was reported. In meat and meat products 2.6% in raw beef (Simon *et al*., 2011) to 69.8% in pork (Bayeleyegn *et al*., 2004) were reported.

Table 2. Prevalence reports of *L. monocytogenes* in specific foods of animal origin in Ethiopia

Studied area	Food item sampled*	Prevalence of *L .monocytogenes*		References
		Food category	%	
Addis Ababa	Retail meat and milk products	Pork, Ice cream, Minced beef, Fish, Chicken, Cottage cheese	69.8 43.5 47.5 18.6 15.4 1.6	Bayeleyegn *et al*., 2004
Not specified	RTE foods and raw meat products	Ice cream Cakes Soft cheese Meat products	11.7 6.5 3.9 5.1	Mengesha *et al*., 2009
Addis Ababa	Foods of animal origin	Raw milk Whole egg Raw beef Cottage cheese	13 4.3 2.6 1	Simon *et al*., 2011
Addis Ababa and its surrounding town	Retail meat and dairy products	Raw meat Cake	18.3 3	Firehiwot *et al*., 2013
Addis Ababa	Sheep meat from abattoir and butcher shops	Abattoir sample butchers' shop	2.1 5.5	Selamawit, 2014
North Shewa	Raw milk and dairy products	Raw milk Cottage cheese Curdled-milk	7.8 3.1 2.3.	Tefera, 2014
Central Highlands of Ethiopia	Raw milk and milk products	Raw milk Milk products	18.9 36	Eyasu *et al*., 2015
Gondar town	RTE foods of animal origin	Raw meat Minced beef Fish Pizza Raw milk Ice cream Cream cakes	6.66 12 6 8.3 4 15 10.7	Legese *et al*., 2015
Bishoftu and Dukem	Foods of animal origin	chicken and yogurt cottage cheese raw milk in raw beef	3.3 5 8.7 11.2	Sintayehu, 2017
Debre-Birhan Town	Raw bovine milk	dairy producers from vendors	8.6 13	Yeshibelay and Abebe, 2018

*RTE = Ready to Eat-Food

2.7. Detection Methods

2.7.1. Conventional Methods

2.7.1.1. Isolation of *Listeria monocytogenes* from samples

The isolation and identification of *L. monocytogenes* from food, environmental samples and animal specimens require the use of selective agents and enrichment procedures that keep the levels of contaminating microorganisms to reasonable numbers and allow multiplication of *L. monocytogenes* to levels that are enough for detection of the organism. A number of selective compounds that allow growth of *L. monocytogenes* at normal incubation temperatures have been incorporated into culture media, thus shortening the time required for selective growth of the organism. Examples of these selective compounds include cycloheximide, colistin, cefotetan, fosfomycin, lithium chloride, nalidixic acid, acriflavine, phenylethanol, ceftazidime, polymixin-B and moxalactam(OIE, 2008).

There was no single procedure can be credited with being sensitive enough to detect *L. monocytogenes* from all types of food (Donnelly, 1999). In addition, sub-lethally injured *L. monocytogenes* cells can be found in processed food due to freezing, heating, acidification and other types of chemical or physical treatment. These sub-lethally injured bacteria require special culture conditions for damage repair, before being able to be detected in culture (OIE, 2008).

According to OIE (2008) Conventional methods for the isolation of *L. monocytogenes* from food that have gained acceptance for international regulatory purposes include the United States Food and Drug Administration (FDA) method, the Association of Official Analytical Chemists (AOAC) official method, the ISO 11290 Standards, the United States Department of Agriculture (USDA)-Food Safety and Inspection Service (FSIS) method and the French Standards. Conventional methods for the detection of *L. monocytogenes* in food involve selective culture

enrichment with subsequent culturing on selective media, followed by biochemical tests for species identification.

According to majority of the regulatory agencies requirement, the isolation methods must be able to detect *Listeria* organism per 25g of food. Enrichment methods are required, due to sensitivity, to allow the organism to grow and reach a detectable level of 10^4–10^5CFU ml-1, before plating onto selective media and confirmation of cultures. The most common selective gents such as acri avine(inhibit Gram-positive bacteria), nalidixic acid(inhibit Gram-negative bacteria), and cycloheximide (inhibit fungi) and also broad spectrum antimicrobials(ceftazidime, moxalactam and lithium chloride) are employed in enrichment and plating media to suppress competing microorganisms as *Listeria* cells are slow growing and can be rapidly out-grown by those competitors (Law *et al.,* 2015).

The success of detection protocols depends on: (1) the number and the state of the micro-organisms in the sample, (2) the selectivity of the media (a balance between inhibition of competitors and inhibition of the target organism), (3) conditions of incubation (time, temperature, presence of oxygen) and (4) the selectivity of the isolation medium (the ease of distinction between the target organism and competitive microflora) (Aurora *et al.*, 2008).

Selection of more suitable method than others will depend on the nature of the sample taken. ISO Standard 11290, parts 1 and 2 (ISO 11290-1, 2014), can be used for the detection of *L. monocytogenes* in a large variety of food and feed products. Although they recognize that this standard might not be appropriate in every detail in certain instances, they recommend that every effort should be made to apply this horizontal method as far as possible. The FDA and AOAC methods can be used for milk and dairy products. The USDA-FSIS method is recommended for red meat and poultry (raw or cooked ready-to-eat), eggs, egg products and environmental samples. ISO Standard 11290, part 2, applies for the enumeration of *L. monocytogenes,* as well as optional protocols mentioned in the FDA and USDA-FSIS methods.

Food samples intended for analysis must be representative of the food, including the outer surface and the interior. The conventional culture methods include an enrichment procedure based on the use of liquid culture media containing selective agents. The nature of the media and the selective agents vary with the method. Both the FDA and the ISO methods include a pre-

enrichment step that is intended for the recovery of sub-lethally injured *L. monocytogenes* cells, where as in the USDA-FSIS and the AOAC methods, the samples are processed directly into enrichment broth. In the case of the FDA method, the pre-enrichment is carried out at 30°C for 4 hours in Trypticase–Soy Broth containing Yeast Extract (TSB YE) without selective agents.

In case of ISO protocol uses a 'primary enrichment' for 24 hours at 30°C in the presence of selective agents, but at half the concentration (half Fraser broth). Samples are enriched for 24 to 72 hours at 30°C, 35°C or 37°C, depending on the method. For the secondary enrichment, Fraser broth, containing the selective agents at full concentration will use (OIE, 2008).

The FDA method uses bu ered *Listeria* enrichment broth (BLEB) selective enrichment media and TSB YE containing acriflavine, nalidixic acid and cycloheximidefor the isolation and identification of *L. monocytogenes*. The USDA-FSIS method uses two enrichment steps: The 'primary' enrichment is done in University of Vermont medium (UVM), containing nalidixic acid and acriflavine; the 'secondary' enrichment is carried out in Fraser broth, containing nalidixic acid, lithium chloride and acriflavine. The AOAC method calls for selective enrichment in tryptone soy broth containing acriflavine, nalidixic acid and cycloheximide (selective enrichment medium) (Law *et al.,* 2015).

After selective enrichment, cultures are then plated on to selective/differential agar plates for isolation of presumptive colonies of *L. monocytogenes*. All methods, except that of the ISO standard, use Oxford agar or a modification, MOX agar (USDA-FSIS). Oxford agar contains lithium chloride, cycloheximide, colistin, acriflavine, cefotetan and fosfomycin as selective agents, and typical colonies of *Listeria* species are small, black and surrounded by a black halo. Oxford agar was used for the isolation of *L. monocytogenes* from clinical specimens and also used in many studies forthe isolation and detection of *L. monocytogenes* from various food samples. In addition to Oxford agar, the FDA includes LPM or PALCAM agar, which contains lithium chloride, polymixin B, acriflavine and ceftazidime. The MOX agar, used in the USDA-FSIS method, contains lithium chloride, colistin and moxalactam.

The two selective plating media used in the ISO standard method are: agar *Listeria* according to Ottaviani and Agosti (ALOA), which contains lithium chloride, nalidixic acid, ceftazidime, polymyxin B and amphotericin B (or cycloheximide), and any other selective medium, of each laboratory's choice, such as Oxford or PALCAM. Typical colonies of *L. monocytogenes* in ALOA agar are green-blue surrounded by an opaque halo. Other chromogenic media developed to differentiate *L. monocytogenes* colonies from those of other *Listeriae* by CHROMagar are Oxoid chromogenic *Listeria* agar (OCLA), and CHROMagar *Listeria* (Law *et al.,* 2015).

2.7.1.2. Biochemical tests for Identification

Typical *Listeria* species colonies on selective and differential agar plates are then selected for further identification to the species level, using a marker tests such as Gram-staining reaction, catalase test, motility test, β-haemolysis and carbohydrate utilization test. The Christie–Atkins–Munch–Peterson (CAMP) test is useful in identifying the species of *Listeria* isolates. It is used in the ISO and AOAC protocols and it is considered to be optional in the FDA and USDA-FSIS methods. It consists of streaking a ß-haemolytic *Staphylococcus aureu* and *Rhodococcus equi* in single straight lines in parallel, on a sheep blood agar plate or a double-layered agar plate with a very thin blood agar overlay. The streaks should have enough separation to allow test and control *Listeria* strains tobe streaked perpendicularly, in between the two indicator organisms, without quite touching them (separated by 1–2 mm) (OIE, 2008).

After incubation for 24–48 hours at 35–37°C positive reaction consists of an enhanced zone of ß-haemolysis, at the inter-section of the test/control and indicator strains. *Listeria monocytogenes* is positive with the *S. aureus* streak and negative with *R. equi*, whereas the test with *L. ivanovii* gives the reverse reactions (OIE, 2008).

2.7.2. Molecular Methods

As molecular methods are accurate, sensitive and specific, they are increasingly used in identification of *L. monocytogenes* from foods. Various molecular methods used are DNA

hybridization, polymerase chain reaction and real time PCR (RT PCR).Among these, PCR and real time PCR are now established methods for identification of *Listeria monocytogenes* from other non-virulent *Listeria* spp. from foods. The real-time PCR is a very sensitive and quantitative method for direction of pathogen and thus has emerged as most important tool for *L. monocytogenes* detection and quantization in foods (Law *et al*., 2015).Molecular methods have been used, Due to the recent advances in molecular technology, as an alternative to culture and serological methods for food testing. Nucleic-acid based molecular methods detect the pathogen present in food based on the detection of specific DNA or RNA sequences in the target pathogen. These genetic methods can provide highly accurate and reliable results as compared to phenotypic methods, but these molecular methods require specialized instruments and highly trained personnel.

2.8. Antimicrobial Resistance Reports of *Listeria monocytogenes*

Infections caused by drug-resistant bacteria, due to the multiplicity of antibiotics, did not represent a medical problem until the early 1980s. However, evolution of bacteria towards resistance has been considerably accelerated by the selective pressure exerted by over prescription of drugs in clinical settings and their heavy use as growth promoters for farm animals. Since bacteria have the remarkable ability to develop resistance to every antibiotic, we can anticipate that even bacterial species such as *Listeria*, which are still considered to be susceptible to almost all antibiotics, will evolve toward multi resistance (Emmanuel and Patrice, 1999).

Bacterial resistance to conventional antibiotic therapies can possibly increase the number of food-borne infections, which lead to the emergence of more virulent pathogenic microorganisms including *L. monocytogenes*. Emergence of antimicrobial resistance in pathogens from foods, environments, humans, and animals has led to increased number of surveillance programs to monitor the antibiotic resistance profiles of important pathogens (Kuan *et al*., 2017).

With the exception of natural *in-vitro* resistance to older quinolones, fosfomycin, and expanded-spectrum cephalosporins, *L. monocytogenes* is widely susceptible to clinically relevant classes of antibiotics active against Gram-positive bacteria. The reference treatment is currently based on a synergistic association of high doses of aminopenicillin (ampicillin or amoxicillin) and gentamicin. Although rifampin, vancomycin, linezolid, and carbapenems have been proposed as possible alternatives, trimethoprim is generally used in case of intolerance of beta-lactams (Emmanuel and Patrice, 1999).

Ampicillin, gentamicin, and trimethoprimsulfamethoxazole were found to be effective against *L. monocytogenes* with the high susceptibility of 100%, 91.4% and 84.5%, respectively. Thus ampicillin alone or in combination with gentamicin is used as the first-line drugs for the treatment of severe listeriosis (Kuan *et al*., 2017).

Listeria monocytogenes rarely develops acquired resistance to antibiotics, however, recently reported an increased rate of resistance to one or several clinically relevant antibiotics in environmental isolate and less frequently in clinical strains. While, increased rate of resistance by this pathogen remains a marginal phenomenon for clinical strains, although only a limited number of studies have focused on the evaluation of antimicrobial resistance in *Listeria* (Kuan *et al*., 2017).

3. MATERIALS AND METHODS

3.1. Description of the Study Area and Farm

The study was conducted in the Haramaya University Dairy Farm, Eastern Hararghe Zone of Oromia Regional State, Ethiopia, at 17 Kilometers from the city of Harar, and 5km from Haramaya town and in general at approximately 509 kms East of Addis Ababa city. Geographically this area lies at $9^0$26'N latitude and $42^0$3'E longitude, at an altitude of 2000 meters above sea level. The area has a mean temperature ranging from 10 to18 ^{0}C with relative humidity of 65% and an average 800mm annual rainfall. The study farm has a total population of 167 including lactating cows, dry cows, heifers and calves. During this study period, there were 30 lactating cows which were milked 2 times/day at the morning and afternoon. The average milk production of the farm was 600 Liters/day. The milk is supplied daily to student's cafeteria, staff lounge, staff members of the University and other surrounding community. The cows are milked 2 times/day at the morning and at afternoon in milking parlor of five (5) milking machines and sometimes by hand. Routine washes of the milking machine, bulk milk tank, milk harvesting equipments and milking parlor operation area were carried out after each milking terms using cold tap water. Teat drying towel were used for each cows to clean teats before milking. Milkers also wash there hands before starting milking between milking intervals.

3.2. Study Design and Sampling Methods

A cross-sectional study was conducted to detect possible sources of *L. monocytogenes* and other *Listeria* species along Haramaya university Dairy Farm operational stages and sampling locations from December 2017 to May 2018. Including all of 30 lactating dairy cows and all the operational stages were sampled. According Adem *et al*. (2016) on hygienic survey from farm to milk supply stage using *E. coli* in the same farm reported the absence of good farming practices, good hygienic practices and good management practices application with the high possibility of milk contamination from various sources. Considering the identified dairy operational stages, sampling points and available farm management conditions, samples were collected from six

dairy operational stages consisting 14 sampling points with randomly allocated sample number. Overall 200 samples were collected. Thus, the sampling methods, samples from the lactating cows, silage feed and environmental samples which could possibly act as sources of *L. monocytogenes* and other *Listeria* species were collected during each sampling occasions from every locations (Table 3).

Table 3. The Haramaya university dairy farm operational stage, sampling location and number of collected samples.

Dairy sampling stages	Sampling Points	Sampling locations	No. of samples
Silage feed and water	1	Silage	30
	2	Drinking water	10
Cow barn	3	Floor swab	10
Lactating Dairy cows	4	Milk sample from teats	30
Milking operation	5	Towel swabs	10
	6	Bulk milk collectors	10
	7	Milk harvesting cylinder	10
	8	Cleaning water	10
	9	Milking machines canal	10
	10	Milking barn floor	10
	11	Milkers hand swabs	10
Milk auditing	12	Pooled Milk at collection	20
Product supply	13	Pooled Milk at supplyMilk	20
	14	Milk measuring easuring equipment	10
Total			200

3.3. Sample Collection and Sample Type

The samples were aseptically collected from 14 sampling points of six sampling stages which includes silage feed swabs, environmental samples (cow's barn, water samples, milking machine canal swab, teat drying towel, milking parlor floor swabs, milk harvesting cylinder swab, milkers hands swab, bulk milk collectors swab, measuring equipment swab), raw milk samples from (directly from cow teats as one, pooled milk from bulk tank at collection and

pooled milk from bulk tank at supply) were aseptically collected. A swab sample from the four canals of one milking machine was taken as one swab (i.e the 4 milking machine canal as one swab sample).

About 25ml of the raw milk samples from the four teats of a cow were sampled into one universal bottle aseptically as one sample. Pooled milk sample was also taken from bulk milk tank at collection and public supply. Water samples was also collected from dairy cows drinking water and from water used for cleaning purposes.

Sterile buffered peptone water (BPW) and wet cotton swab were used for each swab samples. In case of taking swab samples, including Silage feed, a sterile stick of cotton fitted tip was soaked in buffered peptone water and the rubbed against selected sampling site and then placed in a test tube containing 10ml of BPW. Then all samples were transported to Microbiology Laboratory, College of Veterinary Medicine, Haramaya University, using cold Ice box. Microbiological examination was conducted with in 24 hr of collection according to ISO (11290; 2004).

3.4. Detection Methods

3.4.1. Isolation

A number of methods are available for the detection of *Listeria* species. But considering inclusive sample types, the horizontal method for the detection and enumeration of *L. monocytogenes* in food and animal feed was preferred for this research. ISO 11290-1:1996 is a method for the detection of *L. monocytogenes,* while ISO 11290-2:1998 is a method for enumeration. Both methods are amended in 2004 by including modified media (ISO, 2004a).

For the initial suspension, the selective primary enrichment medium as procedure described by ISO 11290 (Half Fraser Broth) was used as dilution fluid. In general, to prepare the initial suspension, xg or xml of test portion was added to 9x ml or 9x g of the selective primary enrichment medium to obtain a ratio of test portion to selective primary enrichment medium of 1:10 (mass/volume or volume/volume) (ISO, 2004a). See Annex 1.

3.4.1.1. Primary Enrichment

Primary enrichment is a selective enrichment that contains a selective liquid media with reduced concentrations of selective agents and the medium called half Fraser broth. It is usually a pre-enrichment culture in a liquid medium (Half-Fraser broth). Twenty-five milliliters (25ml) of milk and water samples were added to prepared 225 ml of half Fraser broth while a swab samples were left in test tube containing 10ml buffered peptone water. According to ISO (2004) the mixture was homogenized and the test portion was incubated at 30ºC for 24 hours.

3.4.1.2. Secondary Enrichment

Fraser broth medium with full concentration of selective agents was employed. Then 0.1ml of the culture in primary enrichment (regardless of its color) was transferred to a tube containing 10ml of secondary enrichment medium (Fraser broth) (Annex 3). Then the selective enrichment medium (Fraser broth) was incubated at 37 °C for 48 hours.

3.4.1.3. Plating out and Identification Media

Loop full of the culture from the secondary enrichment culture, Fraser broth, was taken and streaked onto sterile PALCAM (polymixinacriflavine lithium chloride ceftazidine aesculine mannitol) agar plates and Oxford agar plates and incubated at 37ºC for 48 hours to determine the presence or absence of *Listeria* species based on the growth of characteristic presumed colonies.

Presumptive colonies of *Listeria* species in general are small, smooth, translucent, and bluish gray when viewed in normal light, but blue green is visible by oblique light and after 48 hours colonies become darker, with a possible greenish sheen and are about 2mm in diameter with black halos and sunken centers (ISO 11290-1, 1996).

For confirmation five colonies suspected to be *Listeria* were transferred separately onto pre-dried plates of Trytic Soya Yeast Extract Agar (TSYEA), which allows well-separated colonies to develop. All colonies were taken for confirmation when fewer than five presumed colonies the

plate is then incubated at 37˚C for 24 hours or until growth is satisfactory. Typical colonies were growing on tryptone Soya yeast extract agar (TSYEA). The colonies of 1mm-2mm in diameter were confirmed by doing biochemical tests selected for *Listeria* species identification (ISO, 2004a).

3.4.2. Biochemical Identification

The following biochemical tests with test outcome (Table 4) were used for identification and confirmation of *Listeria* species (Annex 1 and Annex 4).

***Catalase reaction*:** An isolated colony obtained in TSYEA was transferred on to clean and sterile glass slide. A drop of 3% hydrogen peroxide solution was added to isolate on the glass slide. The immediate formation of gas bubbles indicates a positive reaction.

***Gram staining**:* Gram staining was performed on separate colonies taken from TSYEA *Listeria* species was revealed as gram-positive short rods (ISO, 2004a).

Motility: Colonies from TSYEA was taken and inoculated in to motility medium using strait inoculating and was incubated at 25^0C for 48 hours. *Listeria* species were motile by giving a typical umbrella like growth pattern under the sub surface (ISO11290-1, 1996).

Hemolysis test: Sheep blood agar plates were inoculated with isolated colonies taken from TSYEA using an inoculating loop to determine the hemolytic reaction. The colonies were examined after incubation at 37^0C for 24hours. *L. monocytogenes* shows narrow, clear, light zones of ß-hemolysis. *L. innocua* not shows any clear zone of hemolysis around the colony. *L. seeligeri* shows a weak zone of hemolysis and *L. ivanovii* shows a wide clear zone of ß-hemolysis around the colonies.

Carbohydrate utilization tests: *Listeria* species colonies were inoculated in to Tryptone soya yeast extract broth (TSYEB) and were incubated for 24 hours. Each of the carbohydrate utilization broths (rhaminose, xylose, and mannitol) was prepared in test tubes and using Bromocresol purple as an indicator. The inoculi was a culture from TSYEA and incubated at 37^0C for up to 5 days (ISO 11290-1, 1996). Yellow color indicates positive result due to acid

formation and was occur mostly within 24 to 48 hours. When the culture remains purple, it indicates the bacteria did not ferment the carbohydrate and the result was become negative.

CAMP test: was undertaken by using *S. aureus* and *R. equi* (obtained from reference isolates in Haramaya University Microbiology Centeral Laboratory) streaked vertically on sheep blood agar plate and *Listeria* species isolates from Trypticase Soy Agar with 0.6% Yeast Extract (TSAYE) were streaked horizontally without touching the vertical streaks and incubated for 24hours at 37°C. Simultaneously test (suspected isolate) cultures were streaked on blood agar in the same manner as the test culture. The plates then were incubated at 37^0C for 24 hours. *L. monocytogenes* showed an enhanced zone of hemolysis, forming an arrow head towards the *S. aureus* culture, while in case of *L. ivanovii* no formation of a wide clear "arrow head'' hemolysis towards the *S. aureus, L. seeligeri* shows a weak enhanced hemolysis around *S. aurus* streak while *L. innocua*, *L. welshimeri*, and *L. gray* are non-hemolytic and are considered as CAMP test negative (Gasanov *et al*., 2005).

In general, one isolate was regarded as *L. monocytogenes*s if catalase positive, glucose fermenter with acid production but no gas formation, ferment lactose (after 3-5 days of incubation), expressed hemolysis which produces clear zone on blood agar (Beta hemolytic) (Ryser and Donnelly, 2013; Vazquez- Boland *et al.*, 2001). So biochemical tests of *Listeria* species in this research was checked with those reference tests mentioned above and with (Annexes 2-4).

Table 4. Biochemical reactions to identify and differentiate *Listeria* species

Test used		*Listeria* species reactions					
		L. mon	*L. ivanovii*	*L. innocua*	*L. seeligeri*	*L. welshimeri*	*L. grayi*
Catalase reaction		+	+	+	+	+	+
Grams reaction		+	+	+	+	+	+
Motility		Motile	Motile	Motile	Motile	Motile	Motile
Haemolysis		+	++	_	(+)	_	_
Carbohydrate utilization	L-Rhamnose	+	_	V	_	V	_
	D-Xylose	_	+	_	+	+	_
	D-Mannitol	_	_	_	_	_	+
CAMP reaction	*S.aureus*	+	_	_	(+)	_	_
	R.equi	_	+	_	_	_	_

Note: +: positive; ++: strong positive; (+): weak positive; _: negative; V: variable

3.4.3. Antimicrobial Resistance Profile

Antibiotic resistant bacteria pose a growing problem of concern world widely, since the resistant bacteria can be easily circulated in the environment (ISO, 2014). Effectiveness of current treatments and ability to control infectious diseases in both animals and humans may become hazardous. The antimicrobial resistant test profile of *L. monocytogenes* and other *Listeria* isolates were done based on the standard Kirby Bauer method. Selection of antimicrobials was according to frequently used in both veterinary and medical clinical practices, their importance and availability of antimicrobial disks. Since, there is no standard and specific breakpoint interpretation chart for each specific drug types used to test or checking for it. Therefore, for most of the drugs; the results have been interpreted based on the standard breakpoints reported by NCCL's for Gram positive organisms, and only for penicillin, Erythromycin and tetracycline, the actual zone of interpretation chart for *L. monocytogenes* was used (Annex 5).

The isolates were classified in accordance with the guideline of the National Committee for Clinical Laboratory Standards (NCCLS, 2016) as susceptible, intermediate or resistance for each antibiotic (Annex 5) tested. The discs impregnated with (Amoxicillin, Amplicillin, Gentamicin, Streptomycin, Erythromycin, Kanamycin, Penicillin and Tetracycline) were used. Thus, the zone of inhibition around the antibiotic disc was measured with precision Caliper to the nearest 0.1mm.

3.5. Data Management and Analysis

The data obtained from this investigation was entered into Microsoft Excel 2013 and analyzed using STATA-11. The frequencies of *Listeria* species isolated from different sampling points were described by percentages. The association of *Listeria* species occurrence with milk contaminant risk factors at operational stages of the farms was computed using Chi-square (χ^2) statistical test and significance was at $p<0.05$.

4. RESULTS

Out of 200 samples collected from selected six sampling stages, 40 (20%) were positive for overall *Listeria* species of which *L. monocytogenes* was detected in 11(5.5%) of the total.

4.1. Detection of *L. monocytogenes* and other *Listeria* species along the major dairy operational stages

An overall *Listeria* species were detected at all sampling stages with the detection rate ranging from 10% at milk auditing to 30% at cow barn with no significant differences ($\chi^2 = 2.80$; p-value = 0.73). *L. monocytogenes* was also detected at all stages except at milk auditing. *L. monocytogenes* detection rate at operational stages were 2.8%, 6.6%, 7.5% and 10% from milking operation, pooled milk at supply station, feed and watering and cow barn and milk from cow teat respectively (Table 5).

Table 5. Detection of *L. monocytogenes* and other *Listeria* species along the dairy operational stages

Sampling operational stages	No. of samples examined	No. (%) of positives samples		Total positives		
		L. monocytogenes	Other *Listeria*	No. (%)	χ^2	p-Value
Silage and water	40	3(7.5)	5(12.5)	8(20.0)		
Cow barn	10	1(10.0)	2(20.0)	3(30.0)		
Milk cow	30	3(10.0)	3 (10.0)	6(20.0)		
Milking operation	70	2(2.8)	11(15.7)	13(18.6)	2.80	0.73
Milk auditing	20	0	2(10.0)	2(10.0)		
Milk supply	30	2 (6.6)	6(20.0)	8(26.7)		
Total	200	11(5.5)	29(14.5)	40(20.0)		

4.2. Detection and Distribution of *L. monocytogenes* and other *Listeria* species at sampling location

From 200 samples analyzed, from 14 sampling locations; in which 11 (5.5%) *L. monocytogenes* was isolated and other *Listeria* species were also detected from most of the sampling sources except from drinking water supplied to dairy cows and from milker's hand. *Listeria* species were detected from silage(26.7%), cow barn floor (30%) , teat drying towel (30%), milk from cows teat (20%), cleaning water (20%), milking machine canal (20%), pooled milk at collection (20%), milking parlor floor (40%), pooled milk at supply and milk measuring equipment samples. From the 14 sampling sources, *L. monocytogenes* was detected from silage feed, cow barn, milk from cow teat, milking parlor floor, pooled milk at supply and milk measuring equipment. The result of detected *Listeria* species isolates including *L. monocytogenes* at sampling location is indicated below in (Table 6).

Listeria monocytogenes was the dominant and frequently detected with 11(5.5%) followed by 10(5%) *L. innocua*, 6(3%) *L. gray*, 5(2.5%) *L. ivanovii*, 4(2%) of *L. seeligeri* and *L. welsheri* isolates. Except for *L. ivanovii*, silage was found positive for all *Listeria* species isolated in this study. Milk from cows' teat was positive for all isolates of this study except for *L. ivanovii* and *L. seeligeri*. Likewise, milk measuring equipments were positive for all except for *L. innocua* and *L. seeligeri* (Table 6).

Table 6. Detection and distribution of *L. monocytogenes* and other *Listeria* isolates along sampling locations

Sampling stage	No.	Sampling locations	No.of samples	No. (%) of Positives	Distribution of *L. monocytogenes* and other *Listeria* species isolates (%)					
					L. monocytogenes	*L. ivanovii*	*L. innocua*	*L. seeligeri*	*L.welshimeri*	*L.gray*
Feed and watering	1	Silage feed	30	8 (26.7)	3 (10.0)	0	2 (6.7)	1 (3.33)	1 (3.33)	1 (3.33)
	2	Drinking water	10	0	0	0	0	0	0	0
Housing	3	Cow barn floor	10	3 (30.0)	1 (10.0)	0	0	1 (10.0)	0	1 (10.0)
Cow teat	4	Milk from cow teat	30	6 (20.0)	3 (10.0)	0	1 (3.33)	0	1 (3.33)	1 (3.33)
Milking operation	5	Milkers hand	10	0	0	0	0	0	0	0
	6	Milking machine canal	10	2(20.0)	0	0	2 (20.0)	0	0	0
	7	Milk harvesting cylinder	10	1(10.0)	0	1 (10.0)	0	0	0	0
	8	Bulk milk collector	10	1(10.0)	0	0	1 (10.0)	0	0	0
	9	Teat drying towel	10	3(30.0)	0	0	2 (20.0)	1 (10.0)	0	0
	10	Cleaning water	10	2(20.0)	0	0	1 (10.0)	0	1 (10.0)	0
	11	Milking parlor floor	10	4(40.0)	2 (20.0)	0	0	1 (10.0)	0	1 (10.0)
Milk auditing	12	Pooled milk at collection	20	2(10.0)	0	1 (5.0)	0	0	0	1 (5.0)
Milk supply	13	Pooled milk at supply	10	4(40.0)	1 (10.0)	2 (20.0)	1 (10.0)	0	0	0
	14	Milk measuring equipment	10	4(40.0)	1 (10.0)	1 (10.0)	0	0	1 (10.0)	1 (10.0)
Total	Total		200	40(20.0)	11(5.5)	5(2.5)	10(5.0)	4(2.0)	4 (2.0)	6(3.0)

4.3. Antimicrobial Resistance profile of *Listeria* Isolates

Of the tested 40 isolates resistant to tetracycline (55%), penicillin (45%) and amoxicillin (20%) resistant isolates were observed. Likewise 54.5% and 27.3% of *L. monocytogenes* isolates showed resistance to tetracycline and to each of amoxicillin and streptomycin antibiotics, respectively.

Twenty percent (20%) of *L. ivanovii* showed resistance to each of penicillin and gentamycine, but 40% of them showed resistance to amoxicillin with no resistant isolate to other drugs. *L. innocua* showed resistance to each of penicillin and tetracycline (60.0%). *L. seeligeri* showed 75% resistance to penicillin as the same for *L. welshimeri*. Again 66.7% of *L.gray* showed resistance to each penicillin and tetracycline.

However, *Listeria* species isolates were susceptible to Kanamycin (92.5%), erythromycin (87%), each of ampicillin and streptomycin (80%) and each of amoxicillin and gentamycin (70%) were observed. All 100% *L. monocytogene* isolates were susceptible to kanamycin and erythromycin, 90% to penicillin, equally (72.7%) to ampicillin and streptomycin, equally (63.6%) to amoxicillin and gentamycine (Table 7).

Table 7. Antimicrobial resistance profile for *Listeria* isolates

***Listeria* species**	**No. of tested isolates**	**Responses to the drug**	**Isolates and antimicrobial profiles of Listeria Spp.**							
			K	P	E	AMP	AMX	T	S	G
L. monocytogenes	11	S No. (%)	11(100.0)	10(90.9)	11(100.0)	8(72.7)	7(63.6)	4 (36.7)	8 (72.7)	7(63.6)
		I No. (%)	0	0	0	3(27.3)	1(9.1)	1(9.1)	0	4 (36.4)
		R No. (%)	0	1(9.1)	0	0	3(27.3)	6(54.5)	3(27.3)	0
L. ivanovii	5	S No. (%)	5(100.0)	2(40.0)	4(80.0)	4(80.0)	2(40.0)	1(20.0)	5(100.0)	3(60.0)
		I No. (%)	0	2(40.0)	1(20.0)	1(20.0)	1(20.0)	4 (80.0)	0	1(20.0)
		R No. (%)	0	1(20.0)	0	0	2(40.0)	0	0	1(20.0)
L. innocua	10	S No. (%)	9(90.0)	1(10.0)	10(100.0)	8(80.0)	9(90.0)	3 (30.0)	8 (80.0)	8(80.0)
		I No. (%)	1(10.0)	3(30.0)	0	2(20.0)	1(10.0)	1 (10.0)	0	1(20.0)
		R No. (%)	0	6(60.0)	0	0	0	6(60.0)	2(20.0)	1(20)
L. seeligeri	4	S No. (%)	4(100.0)	0	4(100.0)	4(100.0)	4(100.0)	0	3(75.0)	2(50.0)
		I No. (%)	0	1(25.0)	0	0	0	0	0	0
		R No. (%)	0	3(75.0)	0	0	0	4(100.0)	1(25.0)	2(50.0)
L. welsheri	4	S No. (%)	3(75.0)	1(25.0)	3(75.0)	4(100.0)	1(25.0)	0	4 (100.0)	3(75.0)
		I No. (%)	1(25.0)	0	1(25.0)	0	1(25.0)	2(50.0)	0	0
		R No. (%)	0	3(75.0)	0	0	2(50.0)	2(50.0)	0	1(25.0)
L. gray	6	S No. (%)	5(83.3)	2(33.3)	3(50.0)	4(66.7)	5(83.3)	0	4 (66.7)	5(83.3)
		I No. (%)	0	0	2(33.3)	1(16.7)	0	2(33.3)	1(16.66)	1(16.7)
		R No. (%)	1(16.7)	4(66.7)	116.7)	1(16.7)	1(16.7)	4(66.7)	1(16.66)	0
Total	40	S No. (%)	37(92.5)	16(40.0)	35(87.5)	32(80.0)	28(70.0)	8(20.0)	32(80.0)	28(70.0)
		I No. (%)	2(5.0)	6(15.0)	4(10.0)	7(17.5)	4(10.0)	10(25.0)	1(2.5)	7(17.5)
		R No. (%)	1(2.5)	18(45.0)	1(2.5)	1(2.5)	8(20.0)	22(55.0)	7(17.5)	5(12.5)

K: kanamycin; P: penicillin; E: erythromycin; Amp: ampicillin; Amx: amoxicillin; T: tetracyclin; S: streptomycin; G: gentamycine; S: susceptible; I: intermediate; R: resistant

Of the detected *Listeria* isolates, 33(82.5%) expressed single to MDR. As shown in Table 9, 72.7%, 60.0% and 80.0% of *L. monocytogenes*, *L. ivanovii* and *L. innocua* showed resistance to at least on drug of study. On the other hand all, 100% of each *L. seeligeri*, *L. welshimeri* and *L. gray* showed resistance to at least one drug of study (Table 8).

Of all the 40 *Listeria* species isolates, 14(35%), 10(25%), 7(17.5%) and 2(5%) isolates were resistance to single, two, three and four drugs respectively. Of the *L. monocytogenes*, 5(45.5%), 1(9.1%) and 2(18.2%) have developed resistance to single, two and three drugs respectively (Table 8).

Table 8. Single to multi drug resistance profile of the isolated *Listeria* species

***Listeria* spp**.	**No. of isolates**	**No.(%)Resistance to at least one drug**	**No. (%) Multi Drug Resistance profiles**			
			Single	Two	Three	Four
L. monocytogenes	11	8(72.7)	5(45.4)	1(9.1)	2(18.2)	0
L. ivonovii	5	3(60.0)	2(40.0)	1(20.0)	0	0
L. innocua	10	8(80.0)	3(30.0)	3(30.0)	2(20.0)	0
L. seeligeri	4	4(100)	1(25.0)	1(25.0)	1(25.0)	1(25.0)
L. welsheri	4	4(100)	1(25.0)	2(50.0)	1(25.0)	0
L. gray	6	6(100)	2(33.3)	2(33.3)	1(16.7)	1(25.0)
Total	40	33(82.5)	4(35.0)	10(25.0)	7(17.5)	2(5.0)

The 33 isolates showing resistance against single to combination of two or more drugs for each of the isolate *Listeria* were shown in Table 9.

Table 9. Single to multiple drugs resistance combination profile of the isolated *Listeria* species

Resistance profiles	Drugs combinations	No. of isolates	No. (%) resistant isolates of *Listeria* isolates					
			L. monocytogenes	*L .ivanovii*	*L. inocua*	*L .seeligeri*	*L. welsheri*	*L .gray*
Single drug	AML	3	1 (33.3)	1 (33.3)	0	0	1 (33.3)	0
	G	1	0	1(100)	0	0	0	0
	P	4	1 (25.0)	0	2 (50.0)	0	0	1 (25.0)
	T	6	3 (50.0)	0	1 (16.7)	1 (16.7)	0	1(16.7)
Two drugs	AML-T	1	0	0	0	0	0	1 (100)
	P-T	4	0	0	2 (50.0)	1 (25.0)	1 (25.0)	0
	P-AML	3	0	1 (33.3)	0	0	1 (33.3	1 (33.3)
	T-S	2	1 (50.0)	0	1 (50.0)	0	0	0
Three drugs	AML-T-S	2	2 (100)	0	0	0	0	0
	P-T-G	3	0	0	1 (33.3)	1 (33.3)	1 (33.3)	0
	P-T-S	2	0	0	1 (50.0)	0	0	1 (50.0)
Four drugs	K-P-E-T	1	0	0	0	0	0	1 (100)
	P-T-S-G	1	0	0	0	1(100)	0	0
Not resistant at all		7	3 (42.8)	2 (28.6)	2 (28.6)	0	0	0
Total		40	11 (27.5)	5 (12.5)	10 (25.0)	4(10.0)	4(10.0)	6 (15.0)

K: kanamycin; P: penicillin; E: erythromycin; Amp: ampicillin; Amx: amoxicillin; T: tetracycline; S: streptomycin; G: gentamycine

5. DISCUSSIONS

Listeria species including *L. monocytogenes* is most frequently prevalent in the milk-processing environment including steps, drains and floors (Kells and Gilmour, 2004; OIE, 2014). A recent report indicates a close relationship between onsets of listeriosis in ruminants and feeding of contaminated silage (Donnelly, 2001; OIE, 2014). Due to this relation a high excretion rates of *L. monocytogenes* in milk from asymptomatic cows have frequently been observed at 10%.

Despite the reports of detection and isolation of *Listeria* species including *L. monocytogenes* from foods of animal origin (raw milk and milk products, in raw meat and retail meats) and other ready to eat foods, the present detection and isolation of *Listeria* species from animal feeds silage (26.7%) and other environmental samples within the dairy farms is the first report in Ethiopia, thus, this feed and water contact as its most possible source of infection for dairy cows or contamination to raw milk from these environmental contaminant.

5.1. Detection of *L. monocytogenes* and other *Listeria* species along the dairy farm operational stages

The overall 40 (20%) isolates of *Listeria* species including *L. monocytogenes* were within the range of 14% at Jimma (Muhammed *et al.*, 2013) to 32.9% at Bishoftu and Dukem (Sintayehu, 2017) of central Ethiopia. The present finding is still comparable with 20.88% (Yeshibelay and Abebe, 2018), 22% (Simon *et al*., 2011), 24.2% (Tefera, 2014) from Ethiopia. But, it was lower than the 60% *Listeria* species reported in Uganda (Mugampoza *et al*., 2011).

The 5.5% *L. monocytogenes* isolation in our present finding is comparable with 5.1% *in* retailed raw milk (Bayeleyegn *et al*., 2004), 5.4% (Simon *et al*., 2011; Firehiwot *et al*., 2013) and 5.8% in Ready to eat foods (Sintayehu, 2017), 4.4% in sheep meat (Selamawit, 2014), 4.8% in milk and milk products (Eyasu *et al*., 2015) and 8.84% (Yeshibelay and

Abebe, 2018). The present finding was still similar with the 8.19% raw milk in Ankara (Salibaba *et al*., 2018) and 7.1% dairy operations in U.S by (Kessel *et al*., 2011).

The similarities in *Listeria* isolates in Ethiopia may be due to either similar sample types, laboratory methods applied or hygiene practices and geography of the study areas ranging from farm to foods to the level of supermarkets. There might also be due to transfer and cross contamination with *Listeria* species to the level of milk supply to public stations and within the operational activities in the farm.

Listeria species including *L. monocytogene* were detected at all operational stages. In similar reports from USA (Kessel *et al*., 2011) detected *L. monocytogenes* from dairy farm environments indicating that fecal shedding and environmental contamination of *L. monocytogenes* make contamination of the bulk milk very difficult to avoid and Dairy farms have been identified as a reservoir of *L. monocytogenes*, and there is significant strain diversity within and across farms. Similarly *L. monocytogenes* was isolated from pooled milk sample and from milking operation samples much more frequently than from the dairy cows and their environments due to formation of *L. monocytogenes* biofilms in the milking system and dairy environment acting as a consistent source of the bacterium in the bulk milk.

5.2. Detection of *L. monocytogenes* and other *Listeria* species from sampling locations

From the major six operational stages analyzed, the 14 specific sampling sources, *Listeria* species were detected in 12 sampling sources indicating vast of locations could be possible sources of *Listeria* from milk contamination having public health risk. In contrary, any *Listeria* species were not detected in samples from drinking water and milker's hand.

Although *L. monocytogenes* was the dominant and frequently detected isolate with 5.5%, *L. innocua* (5%), *L. gray* (3%), *L. ivanovii* (2.5%), *L. seeligeri* and *L. welshimeri* (2%) each were observed as contamination of the farm line with diversified *Listeria* species. *L. monocytogenes* and *L. innocua* share the same ecological niche and therefore *L. innocua* could be used as an indicator for the presence of *L. monocytogenes* (Doyle, 2013).

Listeria monocytogenes was detected higher in milking parlor floor (20%) and 10% from each (silage feed, raw milk from cow's teat, milk measuring equipment and pooled milk supply) and barn (10%) indicates that these points were critical control points for corrective action. The present finding of 10% of *L. monocytogenes* in raw milk is slightly lower than the 22% (Simon *et al*., 2011), 18.9% (Eyasu *et al*., 2015), 7.8% (Tefera, 2014) from varies foods in Ethiopia. It was similar with 8.19% from raw milk samples in Ankara (Salibaba *et al*., 2018), 8.8% raw cow milk samples from Ghana (Kwarteng *et al*., 2018), 7.5% from tanker milk samples of reported from Algeria. Poor hygienic conditions during milking, transport, milk storage, infected cow, management practices of cattle feed (poor quality silage) leads to contamination of raw milk with *L. monocytogenes.* The present 20% overall and 3.3% to 10% *Listeria* species from cow teat indicates an asymptomatic cow shedding the pathogen or acts as survival and growth of the pathogen that contaminate the milking machine. This was shown by 20% contaminated milking machine that was believed to be a common source for milk contamination. The *Listeria* species in raw milk from pooled milk samples at collection (10%) and at supply (40%) may be due to the present only single contaminated cow contaminating the others.

Interestingly, it is for the first time that *Listeria* species are detected from silage feed and dairy farm environmental samples in Ethiopia with finding of 26.6% isolates of listeria species were detected from silage feed samples tested. In the environment, these saprophytic microorganisms can live in soil, water and decaying vegetables from which it could contaminate animal feed. Similarly, Ueno, (1996) reported 16.6% *L. monocytogenes* isolates from poor quality silage. Since all *Listeria* species are potential

food contaminants, the presence of any of these species on food stuffs can be considered as an indicator of their contamination and of the potential presence of *L. monocytogenes*.

The presence of *Listeria* species in bulk tank milk and milking machine canal and milking operation indicating a significant risk of contamination not only for the purpose of raw milk and raw milk product consumption but also risk for the processing plants as it persists in the dairy environments and its ability to form biofilms. Therefore, contamination of the raw milk entering the plant is a concern to the processors even when the milk is being pasteurized. Recent reports indicated that *Listeria* can be present in biofilms that form in the milking parlor equipment and bulk tank (Latorre *et al*., 2010).

5.3. Antimicrobial Resistance profiles of *L. monocytogenes* and other *Listera* species isolates

Antimicrobial resistance profiles of *Listeria* species including *L. monocytogenes* were tested for eight antimicrobials. Out of 11 isolated *L. monocytogenes*, 72.7% showed resistance to one or more antimicrobials tested. In this current finding Multiple drug resistance (MDR) profiles for isolated *L. monocytogenes* were; five (45%), one (9%) and two (18.2%) of *L. monocytogenes* isolates were resistance to single, two and three antimicrobials respectively. In line with reports in Ethiopia resistance against tetracycline was the most frequently reported it's frequently prescription as oxytetracyclin in Veterinary Medicine and in tetracycline in human clinical cases or in other mode of actions in Ethiopia (Yeshibelay and Abebe, 2018). The 54.5% resistance of tetracycline in this finding was relatively higher than 25% (Yeshibelay and Abebe, 2018) and lower than the 77.8% resistant reported Selamawit (2014). Unlike the zero reports of Tefera (2014) to streptomycin, amoxicillin and streptomycin resistance (27.3%) were observed in this study in case of *L. monocytogenes*..

Interestingly, those isolates of *L. monocytogenes* developing resistance and multi drug resistant were isolates detected from raw milk samples (teats milk and pooled milk) and

also silage feeds supplied cows. In Ethiopia, reports indicated that 71% to 97% of milk is sold through an informal market. The milk in such cases is either consumed in raw form at home or traditionally processed into other dairy products. In addition, there has not been a pre-set standard for milk quality in the Ethiopia (Eyasu *et al.,* 2015). Repeatedly using of one antimicrobial overlong period of time and unjustified dose can develop antimicrobial resistance *L. monocytogenes*.

6. CONCLUSIONS AND RECOMMENDATIONS

Milk and dairy products have high nutritional value. Hence, these food products are very suitable for development of microorganisms, pathogens including *L. monocytogenes* are commonly found in the dairy environment, on the farm and in the processing plants. *L. monocytogenes* and other *Listeria* species were detected in all those selected six sampling operational stages and in most of sampling locations acting as sources of contamination. This result for the presence of *Listeria* species in analyzed silage feed, environmental samples within the farm and from milk samples indicates the potential for contamination with *L. monocytogenes* and other *Listeria* species as indicators for possible contamination practices. The presence of these bacteria may be attributed to the unclean working environment, poor sanitary conditions of personnel, milking machine canals and equipments used in milking operations. We can draw the conclusion that the detected *L. monocytogenes* in from raw milk from cows teat was due to improperly fermented silage, and the raw milk at bulk tank and at public supply revealed presence of these environmental pathogen, which can pose a threat to human life. The presence of *L. monocytogenes* in sampled milk warrants a need for regulatory mechanism to be set and implemented in operational stages. The study also suggests the need for improved food safety through the implementation of hygienic measures at all levels from milking operation to milk supply to public station.

Listeria monocytogenes may not be seen as potential clinical threat in Ethiopia today, with the increasing trend of transnational spread and emerging diseases. The probable risk that it might pose in the years to come cannot be ignored. Numerous risk factors are associated with favoring for growth and contamination with *L. monocytogenes* in silage feed, in dairy plant operation environments and in milk produced. These factors need to be addressed and considered a serious hazard to identify and control measures for an effective prevention and control program of the pathogen. In addition, addressing communication, risk perception and consumer practices to the public are mandatory. Furthermore, the present study detected widespread resistance of *L. monocytogenes* to

commonly used antimicrobials. In addition, the prevalence of multi-drug resistance of the bacterium is also phenomenon, which gives cause for serious concern.

The poor hygienic conditions during milking and after milked which are not done following the principles of food hygiene were the cause for contaminated milk. The results show that there is the risk infection by *L. monocytogenes* to consumers of raw milk supplied to consumers.

In conclusion, dairy farm hygienic practices and monitoring and corrective actions have to be settled in order to control milk contamination with *L. monocytogenes*. In addition, as explained above *L. monocytogenes* is proved to be sensitive to many and available drugs. So, appropriate drug selection for Listeriosis and checking for effectiveness must be made as routine way in food microbiology laboratories.

Based on these findings, the following points are suggested as recommendations;

- A further detection of *L. monocytogenes* and other *Listeria* species in animal, animal feeds and the dairy farm environments and further contaminating risk factors assessment in Ethiopia.
- Proper silage preparation and maintaining good storage conditions
- Preparation, maintaining and implementing of dairy farm plant guidelines via routine hygienic monitoring measures should be practiced at all possible operational stages.
- Routine mastitis control with proper cleaning of milking operation areas and equipment's used during inter milking intervals were needed.
- Creating public awareness on the zoonotic risk associated with *Listeria* along dairy industry needed.
- Rationale use and monitoring of drugs by selecting appropriate drugs.
- Implementation and application of general preventative measures, good hygienic practices and good manufacturing practices to prevent *Listeria* species.

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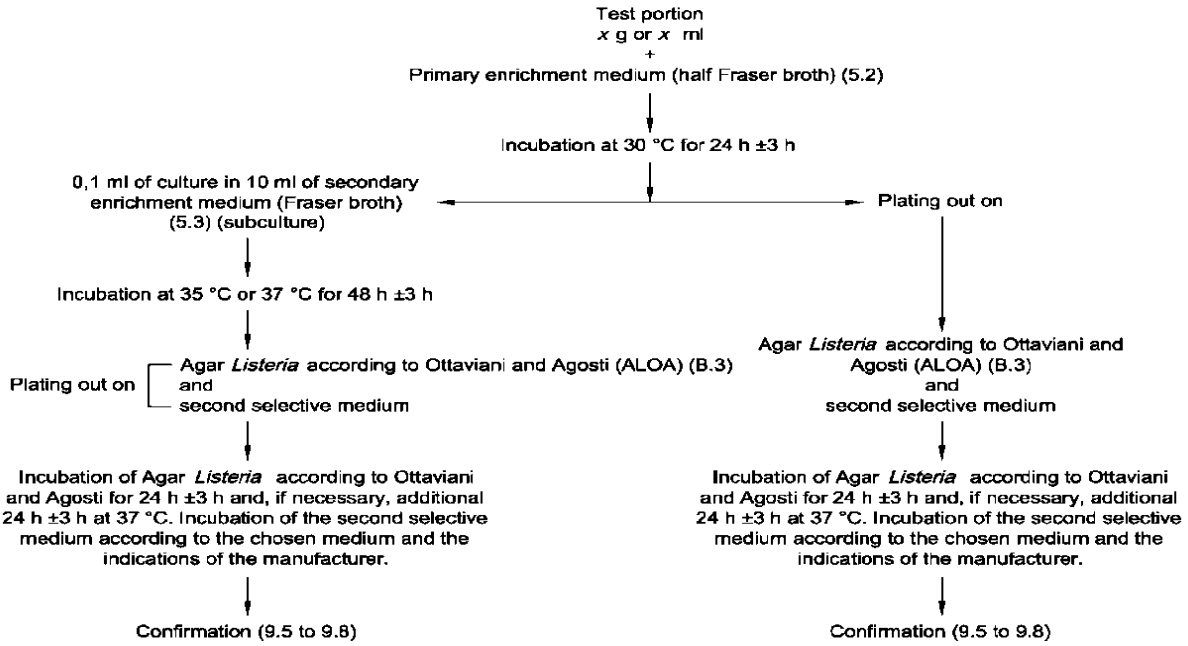
8. ANNEXES

Annex 1. Diagrammatic procedures for *Listeria* characterization

Annex A
(normative)

Diagram of procedure



Source: ISO, 2004

Annex 2. Reactions for the identification of *Listeria* species

Species	Haemolysis	Production of acids		CAMP test	
		Rhamnose	Xylose	S. aureus	R. equi
L. monocytogenes	+	+	-	+	-
L. innocua	-	V	-	-	-
L. ivanovii	+	-	+	-	+
L. seeligeri	(+)	-	+	(+)	-
L. welshimeri	-	V	+	-	-
L. grayi subsp. Grayi	-	-	-	-	-
L. grayi subsp. Murrayi	-	V	-	-	-

V= variable reaction; (+) = weak reaction; + = > 90 % of positive reactions; – = no reaction

Annex 3. *Listeria* selective broth and agar media with their Compositions and Preparations

A. Fraser Broth Base

Code: Himedia M1327

Use: It is recommended, as a primary as well as secondary enrichment medium, for the isolation and enumeration of *Listeria monocytogenes* from food and animal feeds.

Composition	
Ingredients	**Grams / Litre**
Peptic digest of animal tissue	5.000
Casein enzymic hydrolysate	5.000
Yeast extract	5.000
Meat extract	5.000
Sodium chloride	20.000
Disodium hydrogen phosphate.2H2O	12.000
Potassium dihydrogen phosphate	1.350
Esculin	1.000
Lithium chloride	3.000

Final pH (at 25°C) 7.2±0.2

Preparation

Suspend 54.92 grams(equivalent weight of dehydrated medium per litre) 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 for primary enrichment or 1 vial of each to 500 ml medium for secondary enrichment. Mix well and dispense as desired.

B. *Listeria* Selective Agar Base (Oxford Formulation)

Code: CM0856

Use: Selective medium for the detection of *Listeria monocytogenes*.

Composition	
Ingredients	**Grams/Liter**
Columbia Agar Base	39.00
Lithium Chloride	15.00
Esculine	1.00
Ferric-ammonium Citrate	0.50

Final pH 7.2 ± 0.2 at 25ºC

Preparation

- ✓ Suspend 27.75 grams of medium in 500 ml. of distilled water.
- ✓ Heat with frequent agitation until complete dissolution.
- ✓ Distribute into appropriate containers.
- ✓ Sterilize in autoclave at 121°C for 15 minutes. Cool to 50ºC and
- ✓ Aseptically add the contents of 1 via of Listeria selective supplement(SR0140E)
- ✓ Mix well and pour into petridishes

C. *Listeria* Identification Agar Base (PALCAM)

Code: M1064

Use: Selective and differential medium for the diagnosis and detection of *L. monocytogenes*. It is recommended for isolation of *L. monocytogenes* in food products.

Composition	
Ingredients	**Grams/Liter**
Peptone	23.00
Starch	1.00
Mannitol	10.00
Sodium chloride	3.00
Esculin	0.80
Glucose	0.50
Ammonium ferric Citrate	0.50
Dextrose	0.50
Lithium Chloride	15.00
Phenol Red	0.08
Agar	
Final pH 7.2 ± 0.2 at 25ºC	

Preparation

- ✓ Suspend 34.44 grams of medium in 500 ml. of distilled water.
- ✓ Heat with frequent agitation until complete dissolution.
- ✓ Distribute into appropriate containers.
- ✓ Sterilize in autoclave at 121°C for 15 minutes. Cool to 50ºC
- ✓ Aseptically add rehydrated 1 vial of *Listeria* selective supplement (PALCAM) (FD061).

D. Tryptone Soya Yeast Extract Agar (TSYEA)

Composition	
Ingridients	**Gram/Liters**
Tryptone	17.0
Soya peptone	3
Sodium Chloride	5.0
Di potassium phosphate	2.5
Yeast extract	6
Glucose	2.5
Agar	15.0

Preparation

✓ 1 liter of de-ionised water dissolved in 40gm powder and mix well

✓ Heat with frequent agitation and boil for one measure to completely dissolve the powder

✓ Autoclave at 121o C for15minutes

✓ Dispense in to petridishes

E. Carbohydrate Utilization Broth (L-Rhamnose, D-Xylose and D-Mannitol)

I. Base

Composition	
Ingredients	Grams/Liters
Proteose peptone	10 g
Meat extract	1 g
Sodium chloride	5 g
Bromocresol purple	0.02 g
Water	1000 ml

Preparation

- ✓ Dissolve the components in the water, by heating if necessary.
- ✓ Adjust the pH, if necessary, so that after sterilization it is 6.8 ± 0.2 at 25 °C.
- ✓ Dispense the medium into tubes of suitable capacity to obtain appropriate portions of test.
- ✓ Sterilize for 15 min in the autoclave set at 121 °C.

II. Carbohydrate solutions

Composition	
Ingredients	Grams/Liters
Carbohydrate(L-Rhamnose , D-xylose and D-mannitol)	5 g (each)
Water	100 ml

Preparation

Dissolve separately each carbohydrate in 100 ml of water. Then Sterilize by filtration.

III. Complete media

For each carbohydrate, add aseptically x ml of Carbohydrate solutions to 9x ml of the Carbohydrate Utilization Broth base

F. Blood Agar Base

Composition	
Ingredients	**Grams/Liters**
Heart infusion from (solids)	2.0
Pancreatic digest of casein	13.0
Yeast extract	5.0
Agar	15.0
Sodium chloride	5.0

Preparation

✓1liter of deionised water dissolved in 40gm powder and mix well.

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

✓ Autoclave at 121^{o} C for 15 minutes.

✓ Cool the base to 45 to 50^{o}C and add 5% sterile, defibrinated sheep blood

✓ Dispense in to petridishes.

Annex 4. Marker Biochemical tests for Isolation of *Listeria* species and confirmation of *L. monocytogenes*

A. Gram's staining

Procedure

1. Prepare the smear and heat fix.
2. Stain with crystal violet for 60 seconds and rinse with tap water and drain.
3. Flood the slides with iodine and allow remaining 60 seconds and rinsing with tap water and drain.
4. Decolorize with 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 microscopically under oil immersion.

B. Catalase test

Procedures;

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.

C. Haemolysis test

Procedures

1. Isolates colony was taken with an inoculating needle from a typical colony on TSYEA.
2. Streak the sample in to 7% 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).

D. CAMP test

Procedures

1. Take a colony culture with an inoculating needle from a typical colony on TSYEA
2. *Staphylococcus aureus* was taken (CIP: Collection of Institute of Pasteur, 5710).
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 37oC 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 a narrow head towards the *S. aureus* culture.

E. Carbohydrate utilization test

Procedures

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

Annex 5. Antimicrobial resistance profile for *Listeria monocytogenes* by disc diffusion method (Kirby Bauer NCCLS, 2016 and EUCAST, 2018)

Antimicrobials	Zone of inhibition (mm) by disk diffusion method			Remarks
	Susceptible	Intermediate	Resistance	
Amoxicillin	≥ 18	14-17	≤ 13	For Gram positives
Ampicillin	≥ 17	14-16	≤ 13	For Gram positives
Erythromycin	≥ 23	20-22	≤ 20	For *L. monocytogenes*
	≥ 18	14-17	≤ 13	For Gram positives
Gentamycine	≥ 15	13-14	≤ 12	For Gram positives
Kanamycin	≥ 18	14-17	≤ 13	For Gram positives
Penicillin	≥ 34	31-33	≤ 31	For *L. monocytogenes*
	> 16	15	≤ 14	For Gram positives
Streptomycin	≥ 15	12-14	≤ 11	For Gram positives
Tetracycline	≥ 25	22-24	≤ 22	For *L. monocytogenes*
	≥ 19	15-18	≤ 14	For Gram positives