ISOLATION, PHENOTYPIC CHARACTERIZATION AND PUBLIC HEALTH IMPLICATIONS OF *LISTERIA MONOCYTOGENES* CIRCULATING IN SMALLHOLDER DAIRY FARMS OF KOMBOLCHA TOWN AND KUTABER DISTRICT, SOUTH WOLLO ZONE, AMHARA REGIONAL STATE OF ETHIOPIA

MVSc THESIS

By

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

By

Wubshet Nasr Abate

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
ARDO	Animal Resource Development Office
ATCC	American Type Culture Collection
BAM	Bacteriological and Analytical Method
BLEB	Buffered *Listeria* Enrichment Broth
CAMP	Christie Atkins Munch Peterson
CFU	Colony Forming Unit
CLSI	Clinical Laboratory Standards Institute
Csps	Cold Shock Proteins
CVMA	College of Veterinary Medicine and Agriculture
DOA	District Office of Agriculture
DOARD	District Office of Agriculture and Rural Development
FAO	Food and Agricultural Organization
FDA	Food and Drug Authority
GAD	Glutamate Decarboxylase
KADO	Kutaber Agricultural Development Office
KLRDO	Kutaber wereda Livestock Resource Development Office

NCCLS	National Committee for Clinical Laboratory Standards
OIE	Office International des Epizooties
PALCAM	Polymyxin Acriflavine Lithium-chloride Ceftazidime Eesculin Mannitol
PCR	Polymerase Chain Reaction
RTE	Ready to Eat
TSYEA	Tryptone Soya Yeast Extract Agar
USA	United States of America
USDA	US Department of Agriculture-Food and Safety

ABSTRACT

Listeria monocytogenes is a bacterium of veterinary and public health importance, worldwide. Listeriosis in humans is the main food-borne zoonotic illness resulted from consuming dairy and other food products contaminated with mainly *Listeria monocytogenes*. A cross-sectional study was conducted from November 2020 to May 2021 to isolate *Listeria monocytogenes* from raw bovine milk samples, to determine the Antibiogram of isolates and to understand its public health implication in smallholder dairy farms of Kombolcha town and Kutaber district of South Wollo zone, Amhara regional state, Ethiopia. A total of 384 samples were collected from dairy producers using a simple random sampling technique. *Listeria* species isolation was performed, according to standard bacteriological procedures, using Buffered *Listeria* Enrichment broth (BLEB) and Polymyxin Acriflavine Lithium-chloride Ceftazidime Aesculin Mannitol (PALCAM) agar and for confirmation and species identification: carbohydrates utilization, hemolysis, Christie Atkins Munch Peterson (CAMP) and Listeriolysin 0 latex agglutination tests was carried out. The antimicrobial susceptibility test using 9 commonly used antimicrobial drugs against 15 *Listeria monocytogenes* isolates, and a questionnaire survey were also conducted. From the total of 384 samples the overall prevalence of *Listeria* species was 12.8% (49/384) and specifically for *Listeria monocytogenes* was 4% (15/384). In this study, listeriosis is significantly associated with farm management systems and herd size. Based on the antimicrobial susceptibility test, it was found that *Listeria monocytogenes* was sensitive to most drugs except Sulfamethoxazole and nalidixic acid which in both showed 100% resistance. 13.3% of *L. monocytogenes* isolates were also resistant to oxytetracycline, tetracycline, procaine penicillin G and cloxacillin. A structured interview was administered to 70 respondents. Thus, 76.7% of dairy farmers and 20 % of public respondents consume raw milk and 39% of participants were knowledge of zoonotic disease. This presence of *Listeria monocytogenes* in raw milk and its multi-drug resistance pattern is an indication of a serious public health risk. Therefore, creating awareness on milk safety, implementation of milk and personal hygienic practices, implementation of countrywide surveillance and further research to estimate its prevalence both in animals and humans is strongly recommended.

Keywords: *Antibiotic susceptibility, Dairy, Listeria monocytogenes, south Wollo*

1. INTRODUCTION

Listeria species are ubiquitous and they have unique characteristics that permit growth at a freezing temperature which is usually not possible for most food-borne microorganisms (Rocourt *et al*., 2001). *Listeria* can also resist a pH between 4 and 9.6 (Chitlapilly-Dass, 2011). Among the species, *L. monocytogene* is an important cause of human and animal listeriosis. Listeriosis is the main food-borne zoonotic illness because 99% of human infections are resulted from eating food products contaminated with mainly *L. monocytogenes* (Chen *et al*., 2017). It is *a* Gram-positive, rod-shaped, motile, and non-spore-forming bacterium (El-Demerdash and Raslan, 2019) and is primarily known as a veterinary pathogen, which causes basilar meningitis (circling disease) and abortion in sheep and cattle (Aureli, 2001).

Outbreak and sporadic cases of human listeriosis have been associated with contamination of food items like milk, meat, and their products (Sur *et al*., 2012) *Listeria* could ingest with poorly fermented silage which is not acidic enough to kill the bacteria. It also has been ingested through the soil on the grass and placenta from the infected cow. This organism can also vertically transmit from mother to fetus via the placenta and through the infected birth canal (Liu, 2006; Mead *et al*., 2010).

In the USA *L. monocytogenes* human infections are associated with a 94% infection rate and a 15.9% mortality rate (Scallan *et al*., 2011). The mortality rate ranges from 30% to 75% mainly in high-risk groups such as pregnant women, unborn or newly born infants, elderly people, persons with disease conditions like HIV AIDS, and immunocompromised persons were recorded (Liu, 2006; Mead *et al*., 2010).

Unlike infection with other common foodborne microorganisms, it is associated with the highest case of fatality rate (Khan *et al*., 2013). However, for reasons related to lack of awareness of its incidence and lack of detection facilities and inadequate resources together with giving more priorities to other epidemics than listeriosis, its public health significance is not well understood in developing countries including Ethiopia (Molla *et al*., 2004). Raw milk

and milk product consumption are very common in Ethiopia, exposing the public to zoonotic infections including *Listeria* (Amenu *et al*., 2019; Yeserah *et al*., 2020).

Few researchers had isolated *L. monocytogene* from raw milk and dairy products from the central highlands of Ethiopia (Seyoum *et al*., 2015; Girma and Abebe, 2018) and regarding human listeriosis, only one study done in Tigray reported 8.5% of *L. monocytogenes* Prevalence (12/141) among pregnant women (Welekidan *et al*, 2019). The majority of the studies in Ethiopia were conducted with microbiological culture assays by taking milk and meat samples from retail shops in peri-urban and urban areas, therefore being represented only a small fraction of approximately 2% of milk produced in the country (Keba *et al*., 2020). However, with the prevailing informal milk markets, poor hygiene practices and underdeveloped veterinary services, high infection and illness are expected to be prevalent in Ethiopia. The present study is therefore conducted to produce evidence for a better understanding of the epidemiology and public health risk of *L. monocytogenes* pertinent to smallholder dairy farms in the Kombolcha town and Kutaber district of South Wollo zone.

Therefore, the objectives of this study are:

- To isolate *L. monocytogenes* from raw bovine milk samples.
- To estimate the prevalence of *L. monocytogenes* in small-holder dairy farms
- To investigate the susceptibility of isolates to commonly used antimicrobials.
- To determine public health implications of *L. monocytogenes* in dairy farmers and the surrounding community who consumes raw milk and other dairy products.

2. LITERATURE REVIEW

2.1. History and taxonomy of *Listeria*

Listeriosis was first identified in laboratory animals in 1924 in Cambridge (Murray *et al*., 1926). Listeriosis is a disease caused by microorganisms of the genus *Listeria*; occurs worldwide in a variety of animals including man. The link between silage feeding and Listeriosis infection was first recognized in farm animals. In the 1980s listeriosis was known as a foodborne human disease that needed intense research activity (Low and Donachie, 1997). Species of genus *Listeria* consist of; *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. murrayi* and *L. grayi*. They are non-spore-forming, rod-shaped, gram-positive facultative anaerobes, and only *L. ivanovii* and *L. monocytogenes* are pathogenic (Vazquez-Boland *et al*., 2001). Serotypes of *L. monocytogenes* comprise 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7 (McLauchlin, 2008).

2.2. Growth characteristics of *Listeria*

For their optimal growth, *Listeria* species usually require biotin, riboflavin, thiamine, thioctic acid, and different amino acids. Carbohydrates are also vital for *Listeria* multiplication. In general, *Listeria* species grow well on many selective microbiological media designed to support the growth of bacteria. In artificial media, *Listeria* can grow under both aerobic and anaerobic conditions on nonselective media such as tryptone soy broth (TSB) or brain heart infusion (BHI) broth. Tumbling motility can be observed for cultures maintained between 20 and 25°C (McLauchlin, 2008).

2.3. Factors affecting growth and survival of *Listeria* species in foods

2.3.1. Temperature

When the temperature goes below 4°C, most bacteria die or grow poorly, but *Listeria* can live in temperatures ranging from below freezing (-7°C) to body temperature, and it thrives best between -18 and 10°C. Changes in the membrane composition of *L. monocytogenes* are required for survival at these temperatures. Cold shock proteins (CSPs) are produced by *L. monocytogenes* in response to cold acclimation, and they help the bacteria proliferate at low temperatures (Chitlapilly-Dass, 2011). During refrigeration, they acquire well-matched solutes such glycine, betaine, and carnitine. Cold-stressed cells benefit from these solutes because they help them develop. The connection of alternative sigma factories with the core RNA polymerase allows for gene transcription in adverse environmental conditions (Becker *et al.,* 2000). As a result, *Listeria* can be spread by properly freezing ready-to-eat foods. This hazardous bacterium's capacity to flourish in such a wide range of temperature conditions is one of the many hurdles it faces (Ramaswamy *et al*., 2007).

2.3.2. PH

L. monocytogenes is acid-tolerant and may thrive in a wide range of pH settings (4-9.6). At higher temperatures, *L. monocytogenes* becomes more sensitive to acidic environments. During the stomach transit of acidic foods and in the macrophage phagosome, *L. monocytogenes* is exposed to a low pH environment. Several stress adaption mechanisms are used by the pathogen to adjust to and live in these low-pH settings. To survive acid stress, *L. monocytogenes* uses the Glutamate Decarboxylase (GAD) pathway. A tiny percentage of bacteria have a GAD system that plays a big role in their acid resistance. The GAD system transforms extracellular glutamate to extracellular gamma amino butyrate while expending an intracellular proton in a low pH environment. The end result is a reduction in proton concentration within the cell, which alleviates cytoplasmic acidification. Furthermore, gamma amino butyrate is less acidic than glutamate, which aids in environmental alkalization. The

survival of *L. monocytogenes* in stomach fluid is aided by a glutamate decarboxylase pathway (Chitlapilly-Dass, 2011).

2.3.3. Salt and water activity

One of the ways of food preservation employed by the food business is the use of salt to suppress water activity. The capacity of the pathogen *Listeria* to adapt and live in high salt concentrations makes food control challenging (Hill *et al.,* 2002). Listeria's technique for coping with salt stress is a shift in gene expression that results in increased or decreased protein synthesis (Duche *et al.,* 2002). Like most other bacteria, *L. monocytogenes* grows best with a water activity (aw) of 0.90. *L. monocytogenes*, on the other hand, may grow at a (aw) of 0.97 and higher (Saha *et al*., 2015).

2.4. Epidemiology

In comparison to other infectious diseases, listeriosis was a rare occurrence, but it became a devastating foodborne disease in Northern Europe and North America in the 1980s, posing a substantial health danger to people, particularly pregnant women. *Listeria* species have been found in raw milk in various states across the United States, Canada, and numerous European countries (Ryser and Marth, 1991). Starting from 2001, an increase in the number of cases of listeriosis has been seen in England and Wales (Health Protection Agency (London), 2004). Listeriosis is a disease that affects a variety of animals, including sheep, cattle, goats, horses, pigs, camels, dogs, rodents, wild animals, birds, and people. Sheep and cattle are often suffered among them (OIE, 2014). Only isolates belonging to serotypes 1/2a, 1/2b, and 4b were found in 90% of human listeriosis outbreaks, according to epidemiological research, out of 13 serotypes of *L. monocytogenes* (Schuchat *et al*., 1991).

2.4.1. Source of infection and mode of transmission

Animals acquire listeriosis from contaminated surface water, polluted plant, animal feeds, farm walls and water throughs. Animal foods, particularly milk and meat, as well as contact with an aborted fetus and uterine discharge, are the main sources of infection for humans (Heymann, 2004). *Listeria* may also present in poorly fermented silage and infected animal urine (Tewodros and Atsedewoyne, 2012). *L. monocytogenes*, which is frequently prevalent in the feces of cattle, pigs, chickens, turkeys, ducks, crustaceans, and flies, may be asymptomatic in humans and a variety of wild and domestic animals. Similarly, animals may shed these germs in milk for lengthy periods without displaying any illness symptoms (Low and Donachie, 1997). Ingestion of contaminated feed and water is the most typical mechanism of transmission. The infection of pregnant women is primarily caused by the consumption of unpasteurized milk, cheese, and ice cream (Perianu and Bolile, 2004).

2.4.2. Risk factors

Poor nutritional status, sanitary conditions, overcrowding, transportation, and limited access to feed sources are all common risk factors for listeriosis (Galanis and David, 2008). Other risk factors include the organism's massive proliferation in food and the environment, as well as bacteria's capacity to tolerate a wide range of environmental circumstances (Pal and Awel, 2013).

2.5. Pathogenesis

Listeria virulence factors include *ActA*, which is responsible for polymerizing host actin, internalin families which play role in the entry, phospholipases *PlcA* and *PlcB*, and metalloproteases *Mpl* and *UhpT*, which are responsible for sugar uptake (Dussurget *et al*., 2004). Bacteria require *UhpT* to transport glucose-6-phosphate inside the cell, allowing them to grow and multiply. *Listeria* possesses unique virulence characteristics that allow it to

bypass host cells and infect them. Internalin proteins (*InlA*, *InlB*, *InlC*, *InlJ*, *InlH*, and *InlK*) are responsible for gaining access to the host cell through the E-cadherin receptor. As a result, they play an important role in *Listeria* pathogenesis (Bierne *et al*., 2007).

L. monocytogenes can pass through the intestine, the blood-brain barrier, and the placenta. After invading the digestive system, the bacterium enters epithelial cells via phagocytosis, multiplies, and causes infection. *Listeria* harbors host cells through a series of stages that include adhesion, invasion, lysis, proliferation, evasion of host immune mechanisms, and cell-to-cell transmission (Camejo *et al*., 2011).

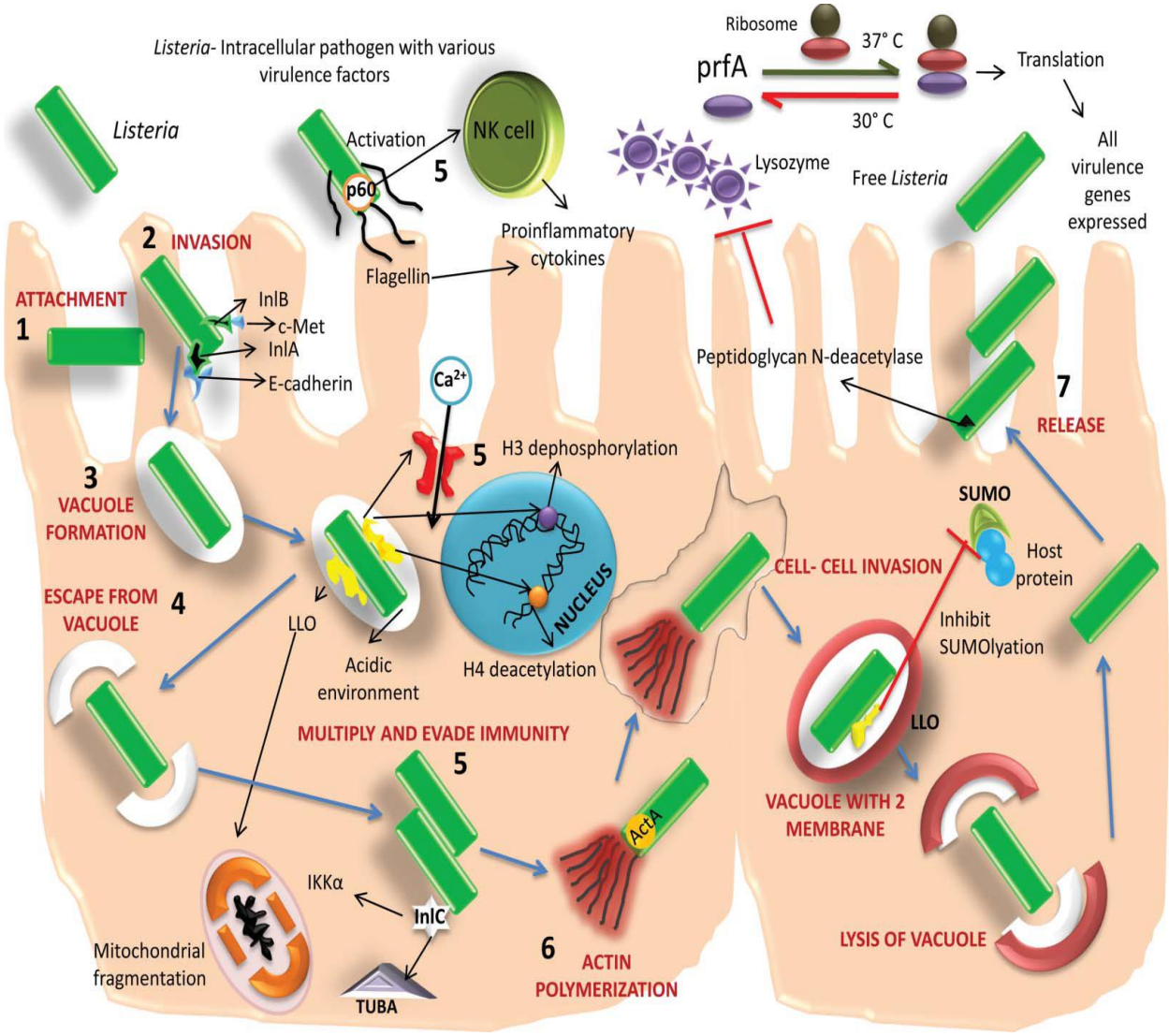


Figure 1: *Listeria* pathogenesis. 1. Attachment to a receptor. 2. Invasion. 3. Phagocytosis. 4. Escaping from the vacuole by numerous mechanisms. 5. Evading the host immunity by diverse mechanisms including *Flagellin*, and *InlC*. 6. The development of the actin tail moves the organism from cell to cell. 7. Finally, the release of the organism (Dhama *et al*., 2015).

2.6. The disease

2.6.1. In animals

Septicemia, encephalitis, and abortion are the most common listeriosis-related illnesses in animals (Malik *et al*., 2002). It causes encephalitis and abortion in sheep and ruminants (Headley *et al*., 2014). Because the animal moves in circles, encephalitis is a type of 'circling' illness. Septicaemic illnesses in horses and pigs occur sporadically. In poultry, listeriosis is uncommon (OIE, 2014).

2.6.2. In humans

In humans, listeriosis can cause septicemia, meningoencephalitis, abortion, and infection in other organs due to foodborne outbreaks (Viswanath *et al*., 2013). The main sources of *Listeria* infection are animal-derived foods such as milk and meat, as well as their by-products (Osman *et al*., 2014). Immunocompromised people, neonates, pregnant women, and the elderly are all susceptible to listeriosis (Sappenfield *et al*., 2013). Septicemia, meningitis, and gastroenteritis are common complications occur in newborns, the elderly and immunocompromised patients, with fatality rates ranging from 30% to 40 (Vera *et al*., 2013).

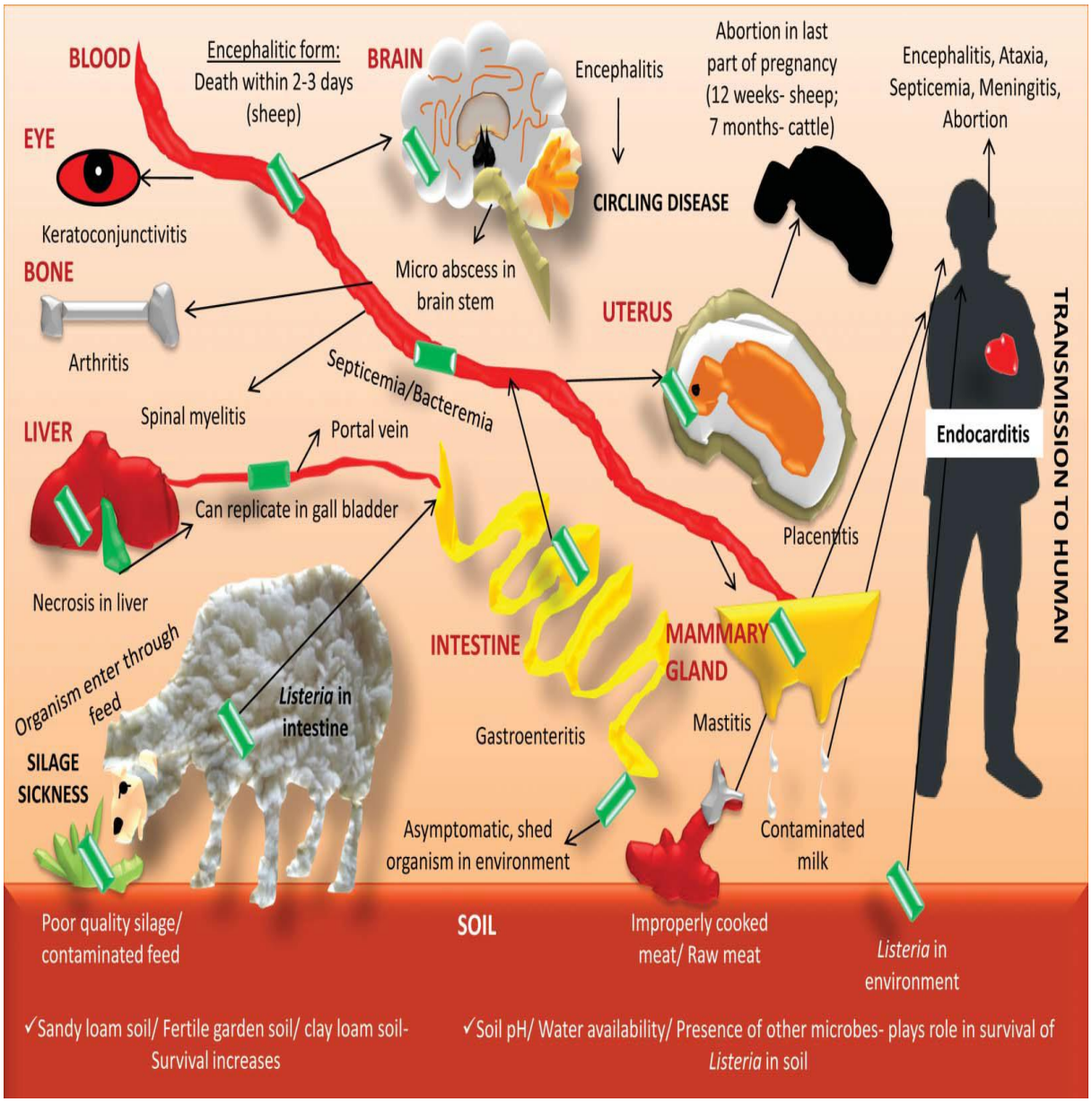


Figure 2: Source of infection and signs of listeriosis in humans and animals (Dhama *et al*., 2015).

2.7. Treatment and prevention

Listeriosis is difficult to treat since it infects many cells. Antibiotics have been used to treat listeriosis in cattle and humans since old times ago. After the appearance of neurological signs in sheep and goats, treatment is frequently challenging. Drugs like penicillin, tetracycline, and

sulphonimides can be used as prophylactics (Radostits *et al*., 2008). Treatment is tough in an immunocompromised host, with a low cure rate. Encephalitis in cattle is treated with chlortetracycline at a dose of 10 mg/kg for five days. Erythromycin and ampicillin are used to treat human listeriosis. Penicillin can also be given every day for seven days with supportive care like rehydration (Staric *et al*., 2008).

Controlling listeriosis is difficult due to the widespread nature of *Listeria*, a lack of quick isolation technologies, the prevalence of an organism in the environment, and a lack of awareness of risk factors other than silage. Cleaning of contaminated materials, avoiding rotten vegetables from contact with animals, hygiene and sanitation measures on farms, avoiding silage in endemic areas, adding tetracycline to animal feeds as a prophylactic, care in the handling of infected animals and the placenta, and routine testing of raw milk are some of the prevention and control measures (Latorre *et al*., 2011).

2.8. Isolation methods

Although the majority of food testing and detection methods rely on culture procedures or antibodies, the food sector is increasingly turning to molecular techniques. When compared to traditional procedures, there are several drawbacks, such as the high cost of materials and reagents, as well as the necessity for highly skilled employees. Thus, an advantage of molecular tests is based on differences within the genome rather than depend on the expression of antigenic factors or enzymes for identification (Gasanov *et al*., 2005).

Because *Listeria* is a slow-growing pathogen that can be cultivated by competitors, bacteriostatic chemicals like acriflavine and nalidixic acid have been added to enrichment media and/or selective agar to prevent competing microflora. Using enrichment approaches, sensitivity can be reached for the detection of *Listeria* organisms in 25 g of food sample (Welshimer *et al*., 1981).

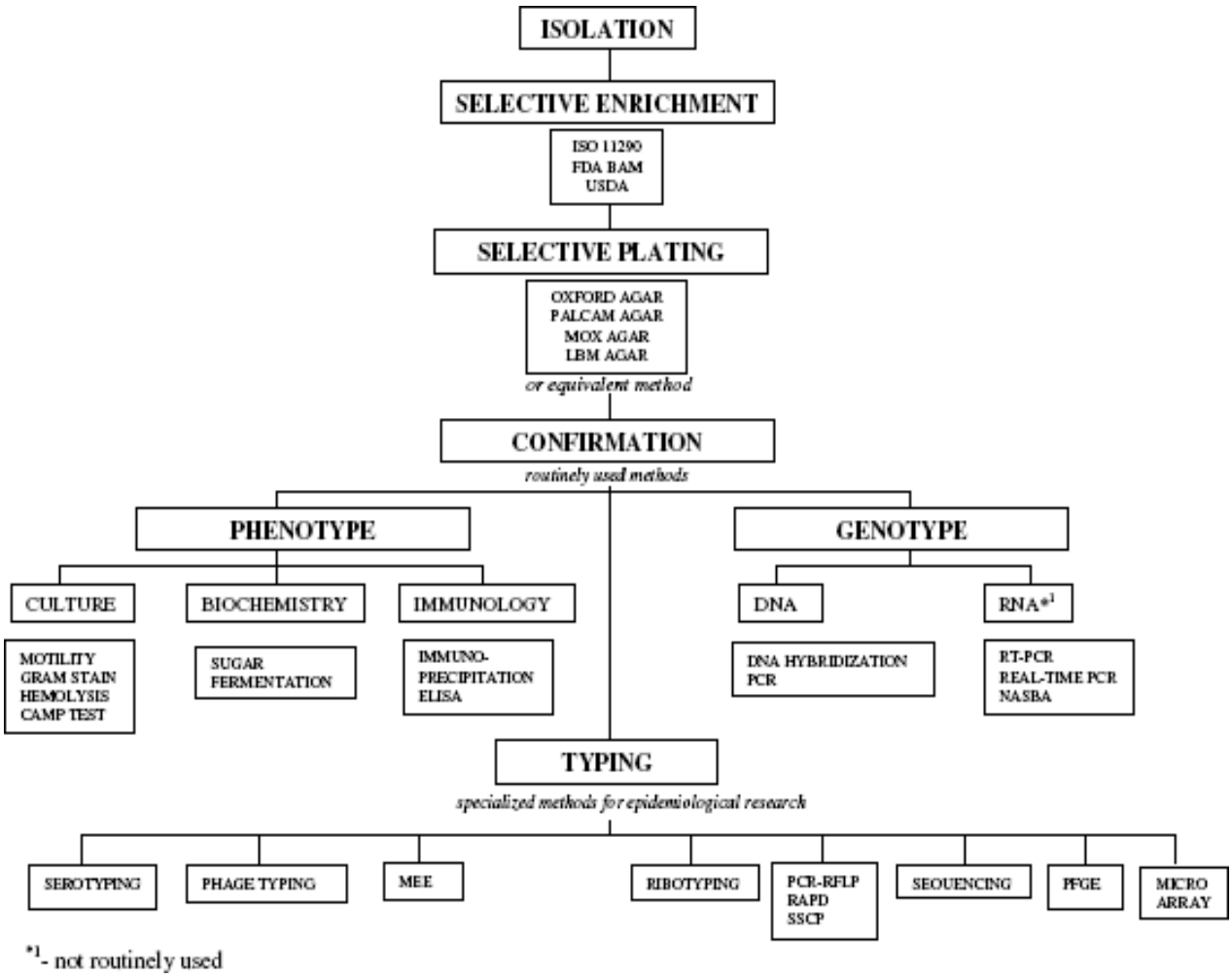


Figure 3: Isolation methods of *Listeria* species from foods and environmental samples.

Source: https://academic.oup.com/femsre/article/29/5/851/547236

2.8.1. Culture techniques

The Bacteriological and Analytical Method (BAM) of the Food and Drug Administration (FDA) (Hitchins and Jinneman, 2013) and the International Organization of Standards (ISO) 11290 method are the most widely used culture reference procedures for the detection of *Listeria* in food samples (ISO, 2004). Both procedures need sample enrichment in selective broth (Gasanov *et al*., 2005).

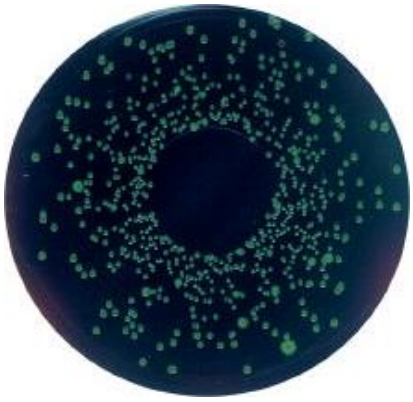
For selective enrichment of *Listeria* in food samples, the FDA-BAM technique recommended the use of buffered *Listeria* enrichment broth (Hitchins and Jinneman, 2013). FDA and ISO recommend PALCAM (Polymyxin Acriflavine Lithium-chloride Ceftazidime Aesculin Mannitol) and Oxford agar as selective differential plating media for *Listeria* species isolation (Zunabovich *et al*., 2011). Typical colonies from selective agar plates are subjected to biochemical assays such as catalase, motility, hemolysis, and carbohydrate utilization tests, as well as the Gram-staining reaction, to characterize *Listeria* to the species level. Specific characteristics of *Listeria are* indicated in Table 1 and characteristics of *Listeria* species in biochemical tests are indicated in Table 2.

Table 1: Specific characteristics of *Listeria* (OIE, 2014)

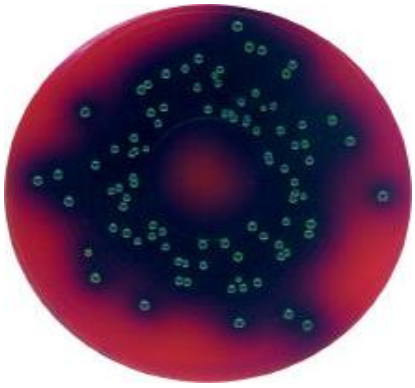
Characteristics	**Reaction**
Gram	+
Morphology	Short, non-spore-forming rods with flagella
Growth	facultative anaerobic, aerobic
Motility	motile at 25 ^{0}C, non-motile at 37 ^{0}C
Catalase	+
Oxidase	-
Hydrolysis	+
Indole	-
Urease	-

Table 2: Biochemical differentiation of *Listeria* species (OIE, 2014)

Species	**hemolysis**	**Acid production**			**CAMP**	
		Rhamnose	**Xylose**	**Mannito**	***S. aureus***	***R. equi***
L. monocytogenes	Positive	Positive	Negative	Negative	Positive	Negative
L. innocua	Negative	Variable	Negative	Negative	Negative	Negative
L. ivanovii	Positive	Negative	Positive	Negative	Negative	Positive
L. seeligeri	Weak positive	Negative	Positive	Negative	Weak positive	Negative
L. welshimeri	Negative	Variable	Positive	Negative	Negative	Negative
L. grayi sub sp. Grayi	Negative	Negative	Negative	Positive	Negative	Negative
L. grayi sub sp. Murrayi	Negative	Positive	Negative	Positive	Negative	Negative



Listeria innocua (ATCC 33090)



Listeria monocytogenes (ATCC19118)

Figure 4: Growth patterns of *Listeria innocua* and *Listeria monocytogenes* on PALCAM agar (Van Netten *et al*., 1989).

2.8.2. Serotyping

Serotyping is a subtyping method for identifying differences in antigens or antigenic components that the immune system recognizes differently (Henriksen, 1978). Antigens expressed on the bacterial cell surface are detected by antisera, which is how serotyping is done. *L. monocytogenes* has long been subtyped using serotyping based on somatic (O) and flagellar (H) antigens. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 are the 13 serotypes of *L. monocytogenes* (Seeliger and Höhne 1979). However, serotypes 1/2a, 1/2b, and 4b account for nearly 95% of strains recovered from human cases and foods (Farber and Peterkin, 1991).

2.8.3. Molecular techniques

Listeria species can be detected using a variety of molecular approaches, including several forms of PCR and loop-mediated isothermal amplification. PCR is a popular technique that uses two single-stranded synthetic primers. The separation of amplified products is achieved via agarose gel electrophoresis, which results in bands visible on the gel (Law *et al*., 2015). *L. monocytogens* virulence indicators genes include *inlA*, *hlyA*, *actA*, and *prfA* (Di Ciccio *et al*., 2012). PCR primers based on the 16S r RNA sequence found in all *Listeria* species are used to create a 938bp amplification product for *Listeria* species detection. In agarose gel electrophoresis, the PCR amplification product is separated and visible on the gel band. Within the PCR amplified 16S r RNA gene and 16S-23S r RNA intergenic space regions, *L. monocytogenes* is distinguished from other *Listeria* species (Goh *et al*., 2012).

2.9. Antibiotics susceptibility

According to previous studies, antimicrobials such as erythromycin, kanamycin, streptomycin, ampicillin, and others are sensitive to *L. monocytogenes* (Aarestrup *et al*., 2007). However, strains of *L. monocytogenes* are resistant to popular antibiotics such as

cephalosporin, clindamycin, sulfamethoxazole (Shen *et al*., 2006) and also to enrofloxacin (Antunes *et al*., 2002). Ampicillin coupled with various aminoglycosides such as gentamicin is the first line of treatment for listeriosis (Motta *et al*., 2004).

2.10. Public health significance

More frequently Pregnant women acquired *Listeria* infection in the third trimester (66%), and in the majority of cases, the sickness of the mother is usually mild with some symptoms like fever and abdominal or back pain or even asymptomatic but in severe cases, it can cause encephalitis, meningoencephalitis, septicemia and abortion (Janakiraman, 2008). The elderly, pregnant women, neonates, and immunocompromised individuals are at the greatest risk of severe listeriosis (Pal, 2007). According to studies, the risk of infection raises with age, with a higher occurrence among the elderly, particularly those over the age of 65. In the United States, pregnant women are roughly 13 times more likely than other healthy people to become infected with *L. monocytogenes* with approximately one in every six (17%) cases occurring during pregnancy. There is a substantial link between invasive listeriosis and lowered T-cell mediated immunity. Listeriosis can strike at any time during pregnancy, but it is most common during the third trimester when cell-mediated immunity is at its lowest. Listeriosis is frequently associated with various debilitating illnesses or disorders, including cancer, organ transplantation, diabetes, liver disease, renal disease, heart disease, HIV infection, and immunosuppressive medication use (Goulet *et al*., 2012).

2.11. Milk production and consumption trend in Ethiopia

Due to rising of population, Milk demand in Etiopia is expected to increase by around 5.5 million tons between 2015 and 2050. Ethiopia's total milk production is 5.6 billion liters per year. The country's entire milk supply equates to a per capita consumption of 56.2 liters of milk (FAO, 2019).

Milk and milk products consumption trends vary based on the volume of milk produced, the production system, market access, and seasonal conditions (fasting in Ethiopian orthodox Christians). According to a study conducted in the Fogera and Metema districts, 20.6 percent and 18 percent of the total milk produced daily is consumed by households respectively, and on average 63 percent is processed into milk products (Tegegne *et al*., 2013). Raw milk intake is widespread throughout the country, particularly in pastoralist communities (Amenu *et al*., 2019).

2.12. The status of the disease in Ethiopia

In Ethiopia, few studies reported the prevalence of listeriosis in milk samples. Seyoum *et al*. reported that the overall prevalence of *Listeria* from raw bovine milk and milk products is 28.4% with an 18.9% prevalence from raw milk in the central highlands of Ethiopia (Seyoum *et al*., 2015). From Addis Ababa, Gebretsadik *et al*. also reported *L. monocytogenes* with an overall prevalence of 26.1% from various food items including raw milk with a high prevalence of 13% (Gebretsadik *et al*., 2011). In Bishoftu and Dukem town of Ethiopia Fisseha reported that 32.9% of the prevalence of *Listeria* species in foods of animal origin, raw milk comprised 33.75% (Fisseha, 2017). Findings of *Listeria* spp. in food samples in Addis Ababa and Gondar towns also reported that the prevalence of 26.6% (Mengesha *et al*., 2009) and 25 % (Garedew *et al*., 2015) respectively. comparatively low prevalence of listeriosis was reported in Jimma and Addis Ababa from raw milk samples 14% and 8.3% respectively (Abera, 2007; Muhammed *et al*., 2013). The most recent study done in Debre-Birhan town reported a 20.88% prevalence of *Listeria* species in row bovine milk collected from smallholder dairy producers (Girma and Abebe, 2018). Regarding human listeriosis, a study done in Tigray reported 8.5% of *L. monocytogenes* prevalence (12/141) among pregnant women (Welekidan *et al,* 2019).

3. MATERIALS AND METHODS

3.1. Descriptions of the study areas

The study was conducted in Kombolcha town and Kutaber District of South Wollo zone, Amhara regional state of Ethiopia. Kombolcha town and Kutaber district are the major milk-producing areas in the South Wollo Zone.

Kombolcha is located 380 km northeast of Addis Ababa. The town has 12 Kebeles with a total population of 143,637, of whom 71,103 were men and 72,534 women. Kombolcha is found at the latitude of 11.083° N and a longitude of 39.733°E with an elevation ranging from 1,842 and 1,915 meters above sea level. It has an annual rainfall ranging from 500 - 900 ml of which 84% is in the long rainy season (June to September) and the dry season extends from October to February. The annual temperature is ranging from 21°C to 36°C. According to the District Office of Agriculture (DOA) report, the district has a total area of 78.6 km^2. About 33.6% of the area is under crop production, and 1.47% is serving as grazing land (DoARD, 2017). Based on the South Wollo zone animal resource development office data, the total cow population in Kombolcha is 9968, and the number of dairy farms that are holding from 3 up to 15 cows is 521. According to the zone's report, moderate and large-scale dairy farms in the Kombolcha town are reached 165 (ARDO, 2020). There was no milk processing plant in the city before but, one company established the plant in mid of this year.

Kutaber is also located in the South Wollo zone. The district is found at 39.031^{o}12" - 39.034^{o}12" E longitude and 11.012^{o}36" -11.018^{o}36" N latitude and poses highland and lowland areas. The average rainfall ranges from 500 to 955ml in the short and long rainy seasons. The area's annual temperature is 22°C on average (KADO, 2018). The main job of the population of the wereda is mixed crop-livestock agriculture. The main livestock reared in the area are cattle, sheep, goats, and equine (KLRDO, 2018). The district has one of the main milk suppliers for Dessie city. Similarly, according to the zone's report, the district has a

livestock population of 74,910 and a cow population of 29674, and the number of large-scale dairy farms is reported as 5 (ARDO, 2020).

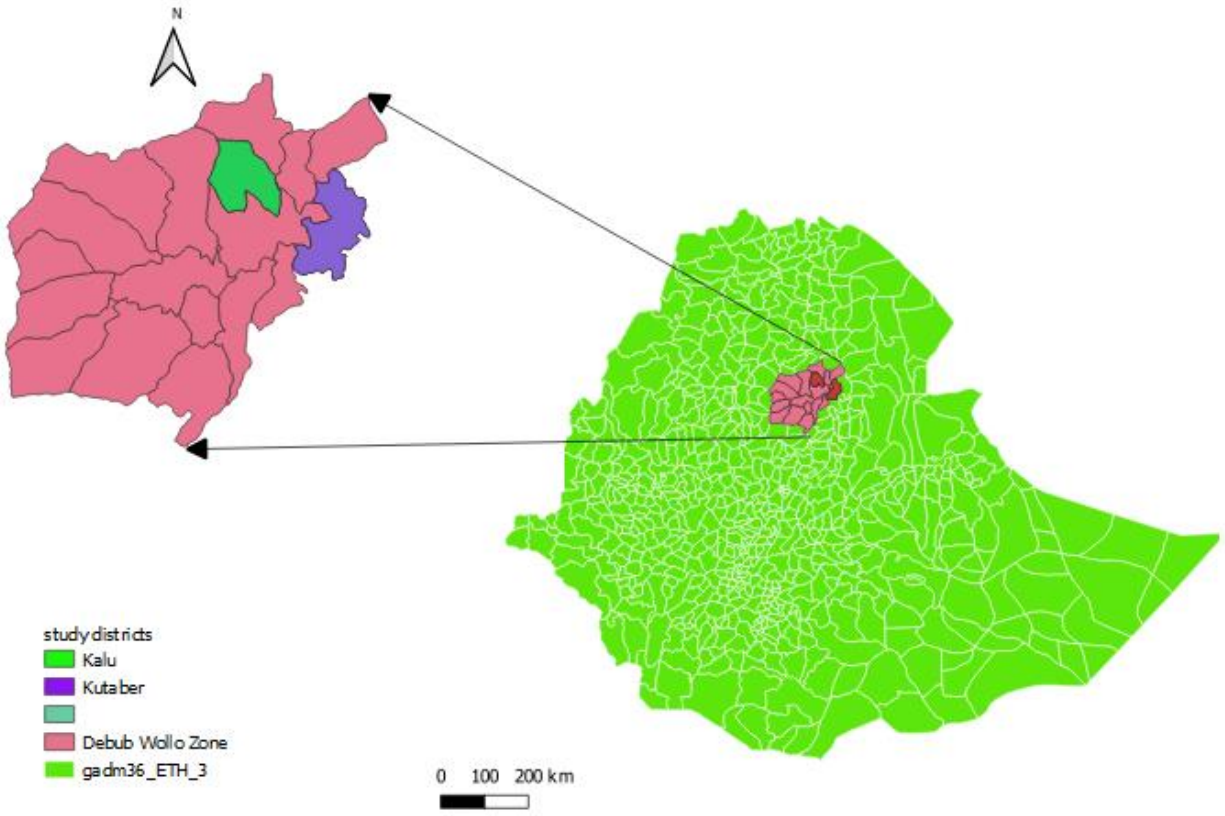


Figure 5: Study area map projected by QGIS

3.2. Study design

A cross-sectional study design was used to collect raw bovine milk samples from selected dairy farms and to undertake laboratory work from November 2020 to May 2021 with objectives of determining the prevalence of *L. monocytogenes*, antimicrobial susceptibility test of isolates and to understand its public health implications.

3.3. Study samples and sample size determination

Dairy farms holding from 3-15 cows were selected for this study. The optimum sample size was calculated by using the formula:

$$n = \frac{1.96^2 \, Pexp \, (1-Pexp)}{d^2}$$

Where 'n' is the required sample size,

'Pexp' is expected prevalence, and'd' is desired absolute precision. Since there was no previous study in the area, according to Thrusfield (2005), by taking 50% expected prevalence with 95% confidence interval and 5% absolute precision. The sample size was calculated as a total of 384 samples (200 of them were from Kombolcha and 184 samples from Kutaber).

3.4. Sampling technique and sample collection

Study farms were selected using a simple random sampling technique from smallholder dairy farms which contained 3-15 cows per farm. Raw milk samples from selected smallholder dairy farms of Kombolcha town and Kutaber district were sampled. All twelve kebeles from Kombolcha town were included. On the other hand, eight kebeles out of twenty-two kebeles of the Kutaber district were selected based on the accessibility of roads and the number of dairy farms. All raw milk samples were collected aseptically using sterile universal bottles. According to Jorgensen 30 ml of raw milk (Jorgensen *et al*., 2005) was collected from milk collection containers of dairy producers and labeled with essential information such as the date of sampling, sample code (Animal ID), and sample source (Annex II). Finally, all samples then immediately transported using an icebox filled with ice to the AAU CVMA microbiology laboratory and enriched with previously prepared BLEB immediately upon arrival.

3.5. Laboratory investigation

3.5.1. Isolation and identification of L. monocytogenes and other Listeria species

Bacteriological culture techniques were applied for the isolation of *Listeria* species from raw milk samples. According to the study done by (Benetti *et al*., 2012), PALCAM agar is preferred to Oxford agar media for isolation of *Listeria* because of its higher selectivity and sensitivity. The techniques recommended by FDA-BAM, ISO 11290, and USDA (US Department of Agriculture-Food and Safety) were used for isolation and identification of *Listeria.*

Primary and secondary enrichment: For achieving high sensitivity, sample enrichment procedures are required to provide an organism to grow into a detectable level (Gasanov *et al*., 2005). According to a suggestion by U.S. Food and drug administration (BAM, 2003), 25 ml of raw milk samples were added to a plastic bag containing 225 ml of Buffered *Listeria* Enrichment Broth (BLEB) (Oxoid, Basingstoke, Hampshire, England). The mixture was then mixed using a laboratory stomacher at maximum speed for 2 minutes. After 4 hours of incubation at 30^{o}C, *Listeria* selective enrichment supplement SR0141 (Oxoid, Basingstoke, Hampshire, England) was aseptically added and the incubation step continued for up to 48 hours at 30^{o}C.

Plating out and identification: Loopful of inoculum from turbidly grew colonies in buffered *Listeria* enrichment broth was taken and streaked into pre-dried sterile plates of PALCAM agar (HIMedia Laboratories Pvt. Ltd. Mumbai-400086, India) prepared after aseptically adding sterile *Listeria* Selective Supplement FD061 (HIMedia Laboratories Pvt. Ltd. Mumbai-400086, India) and incubated at 37^{o}C for 24-48 hours. PALCAM agar is a mixture of 23.0 g/L Columbia Blood Agar, 0.5 g/L glucose, 1.0 g/L starch, 3.0 g/L yeast extract, 0.5g/L aesculin, 0.5g/L Ferric ammonium citrate,10g/L mannitol, 0.08g/L phenol red and 5.0 g/L sodium chloride. The selectivity of the medium is achieved by the addition of lithium

chloride 15 g/L, polymyxin B 0.01 g/L, acriflavine 0.005 g/L, and ceftazidime 0.02 g/L. The isolation on PALCAM agar is based on aesculin hydrolysis and mannitol fermentation (Magalhaes *et al*., 2014). All *Listeria* species hydrolyzed aesculin and manifested by blackening of the medium. Mannitol fermentation is confirmed by a color change in the colony and/or surrounding medium from red or gray to yellow as a result of the production of acid. Typical colonies of grey-green with a black sunken center and a black halo on PALCAM agar (Osman *et al*., 2014) isolated as suspected for *Listeria*.

Confirmation

- **Listeriolysin 0 latex agglutination test (LLOLAT)**

To confirm as *Listeria* species, *Listeria* suspected colonies from PALCAM agar were taken and identified using latex agglutination test with Oxoid *Listeria* test kit (Oxoid, Basingstoke, Hampshire, England). Latex agglutination tests are simple and rapid with 100% sensitivity and specificity (Matar, 1997). The kit contained; *Listeria* latex reagent, *Listeria* positive control antigen 0.5ml**,** 0.85% isotonic saline, preserved with 0.09% sodium azide, and Disposable reaction cards.

LLOLAT was done on clear reaction cards. A drop of saline within one circle on the reaction card was added, a loopful of the suspected bacterial colony was mixed with saline on the reaction card using a sterile mixing stick and then one drop of *Listeria* latex reagent was added, mixed gently using a clean sterilized stick. Finally, agglutination was examined within a maximum of 2 minutes for a positive reaction. Positive colonies were further characterized by using Gram's staining, hemolysis, motility, carbohydrate utilization, and CAMP (Christie Atkins Munch Peterson) tests to confirm as *Listeria* different species (ISO, 1996).

- **Biochemical tests**

Biochemical tests such as hemolysis test, carbohydrate utilization test (fermentation of rhamnose, xylose, and mannitol sugars) and CAMP test were performed for characterization of *Listeria* species.

The CAMP test is used to differentiate CAMP-positive *L. monocytogenes* from other *Listeria* species. The CAMP test was done using *Staphylococcus aureus*. It streaked vertically in a single line across a sheep blood agar plate and streaked *Listeria* isolates horizontal to *S. aureus* streak (Lovett *et al*., 1987). The plates were then incubated at 37^{o}C for 18 to 24 hours. A clear zone of beta hemolysis between the test strain and the culture of *S. aureus* was considered a positive reaction (ISO, 1996).

For the carbohydrate utilization test, carbohydrate utilization broths (rhamnose, xylose, and mannitol) were prepared and sterilized using filter paper in test flasks, and the test was using phenol red as a color indicator. After incubation of the prepared medium with inoculum at 37^{o} C for up to 5 consecutive days, Yellow color indicated a positive result due to acid formation and *Listeria* species showed different characteristics. *L. monocytogenes* showed rhamnose positive, and xylose and mannitol negative (OIE, 2018).

3.5.2. Antibiotic susceptibility test

An antibiotic susceptibility test was performed for *Listeria* isolates by using Muller Hinton Agar (Sisco Research Laboratories Pvt. Ltd. India). The method that was applied for antimicrobial testing is the agar plate antibiotic disk diffusion method, using the Kirby-Bauer technique by 0.5 McFarland Standard (Bauer *et al*., 1966).

Pure colonies of the isolates were taken from nutrient agar and suspended in Muller Hinton Broth dipped using a sterile cotton swab into it and smears uniformly on the prepared Muller Hinton agar, according to the standard procedure, and then the antibiotic discs (Oxoid,

Basingstoke, Hampshire, England) were firmly placed on it, and the plates incubated at 37^{o}C for 24 hrs. Antibiotics discs of commonly used antibiotics such as sulfamethoxazole, tetracycline, oxytetracycline, procaine penicillin G, streptomycin, clindamycin, nalidixic acid, cloxacillin, and erythromycin were used for antimicrobial susceptibility testing.

Finally, the zone of inhibition around the disc was measured using a caliper meter and interpretation is based on criteria of the Clinical and Laboratory Standards Institute (CLSI, 2004) (Annex VII).

3.6. Questioner survey

As a part of the research work, a structured questionnaire was used to assess the farm management and practices relevant to infection and transmission of *Listeria* species in animal and human hosts. Information related to sex, address, educational background, raw milk drinking practice and hygiene practice were collected on a format developed.

Using a structured questioner, dairy producers and randomly selected volunteer participants were interviewed (Annex I). Accordingly, 30 dairy farmers and 40 persons from the community were interviewed. Observational assessments of the farm and milking practice were recorded.

3.7. Data management and analysis

Microsoft Excel was used for data entry and storage and analysis was done by using RStudio Software (Version 1.4.1106 – © 2009-2021 RStudio, PBC). Descriptive statistics were used to describe and process the data. Chi-square statistics also used to compare prevalence between groups and to analyze questioner results. The significance level was established at a 95 % confidence interval.

3.8. Limitation of the study

It was discovered that collecting milk samples from farms was challenging. Due to shortage of time, the current study did not included broad areas and a big number of milk samples. Susceptibility testing was conducted using only available antibiotic discs. Because primers were not available in Ethiopian reference laboratories, molecular characterisation was not carried out. Samples were preserved for future tests if primer importation is possible.

3.9. Ethical declaration

The study was conducted after ethically approved by the AAU CVMA ethical review committee (Date 21/02/2021GC, Ref. No. VM/ERC/19/5/13/2021) and all study work was conducted according to animal research ethics (Annex VII).

4. RESULTS

4.1. Prevalence of *L. monocytogenes* and other *Listeria* species in small-holder dairy farms

From the total of 384 milk samples, the overall prevalence of *Listeria* species was 49 (12.8%). Which comprised 4% (15/384) for *L. monocytogenes*, 4.2%(16/384) for *L. innocua*, 2.1% (8/384) for *L. grayi sub spp. Grayi*, 1.04% (4/384) for *L. grayi sub spp. Murray*, 0.8% (3/384) for *L. seeligeri*, 0.3% (1/384) for *L. welshimeri* and 0.52% (2/384) for unknown *Listeria* species (Figure 6).

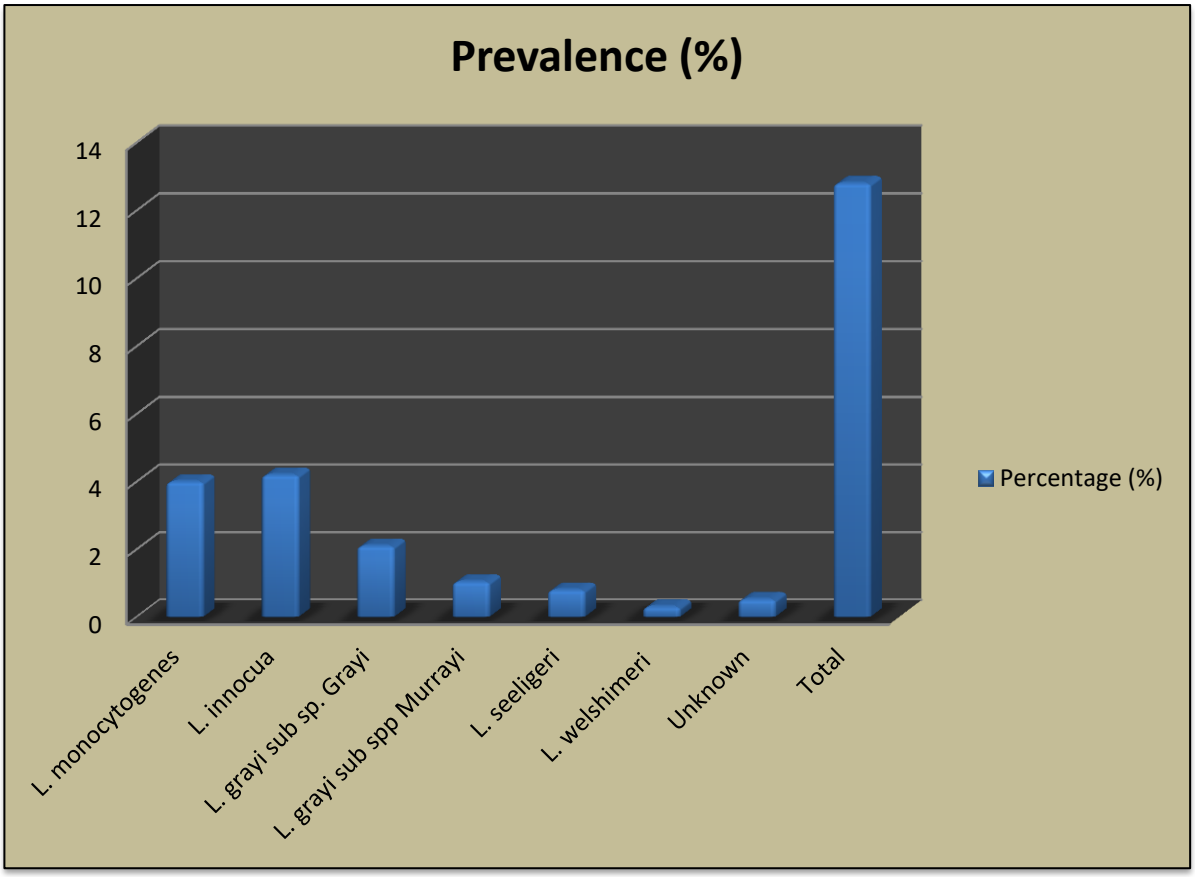


Figure 6: Percentage of *Listeria* species isolated from raw milk samples

4.1.1 Geographical distribution of Listeria species

Considering the overall prevalence of *Listeria*, the high prevalence was found in Kombolcha 13.5% (27/200) and for Kutaber district the prevalence was 12% (22/184). In this case, the difference was not statistically significant (P > 0.5). When taking *L. monocytogenes*, the results were also similar with samples from Kombolcha had the highest prevalence of 4% (8/200) and samples from Kutaber were a prevalence of 3.8% (7/184) (Table 3).

Table 3: Prevalence of *L. monocytogenes* and other *Listeria* species in the study districts

***Listeria* species**	**Prevalence in study districts**		**X^2**	**Odds ratio with 95% CI**	**P-value**
	Kombolcha N (%)	**Kutaber N (%)**	**0.089871**	**0.8701417 (0.4764308-1.589206)**	**0.7643**
L. monocytogenes	8 (4%)	7 (3.8%)			
L. innocua	10 (5%)	6 (3.3%)			
L. grayi sub sp. Grayi	2 (1%)	6 (3.3%)			
L. grayi sub spp Murrayi	2 (1%)	2 (1.1%)			
L. seeligeri	3 (1.5%)	0 (0%)			
L. welshimeri	1 (0.5%	0 (0%)			
Unknown species	1 (0.5%)	1 (0.5%)			
Total	27 (13.5%)	22 (12%)			

N = Number of isolates %= Percentage X^2 = chi-square CI = confidence interval

4.1.2. Prevalence of Listeria in the different farm management systems

Considering the provision of enough and balanced feed, proportionate animals to farm size, relatively clean dairy house and dairy animals, relatively good body condition cows, clean milking equipment and record-keeping practice for good management system and feeding of less nutritious feed, not well-constructed house, no proper management of manure, relatively dirty dairy animals, not properly clean dairy equipment and no record-keeping practice for a poor management system; while taking the overall prevalence of *Listeria* species, the high prevalence was found in poorly managed farms (25.5%) and the low prevalence was found in well-managed farms (4.3%). In this case, the difference was statistically significant ($P < 0.05$). The prevalence of listeriosis in differently managed farms showed in the table below (Table 4).

4.1.3. Prevalence of listeriosis in different herd size

Considering the overall prevalence of *Listeria* species, the highest prevalence was found in farms with 10-15 dairy cows (29%) and the lowest prevalence found in dairy farms with 3-5 cows (5.24%). The difference, in this case, was also found statistically significant ($P < 0.05$) (Table 4).

Table 4: Summary of the prevalence of *L. monocytogenes* isolated in this study

Risk factors		**Total number of samples examined**	**Overall prevalence (%)**	**Prevalence of *L. monocytogenes* (%)**
Study site	Kombolcha	200	27 (13.5%)	8 (4%)
	Kutaber	184	22 (12%)	7 (3.8%)
	X^2		0.089871	0
	p-value		0.7643	1
	OR with 95% CI		3.923353(1.8964-8.1168)	0.9491525 (0.337254-2.671253
Farm management	poor	153	39 (25.5%)	9(5.9%)
	good	231	10 (4.3%)	6(2.3%)
	X^2		14.033	0.057283
	p-value		0.0001796	0.8108
	OR with 95% CI		3.923353(1.8964-8.1168)	1.309645 (0.4569003-3.753924)
Herd size	A	191	10(5.24%)	4(2.1%)
	B	124	19 (15.3%)	3(2.42%)
	C	69	20 (29%)	8(11.6%)
	X^2		36.702	2.5302
	p-value		1.072e-08	0.2822
	OR with 95% CI		3.925234 (1.725926-8.927066)	2.807273 (0.7288633-10.812425)

X^2 = Chi-square, OR= Odds ratio, CI= Confidence interval

4.2. Antibiogram of *L. monocytogenes* isolates

Out of the total of 15 *L. monocytogenes* species subjected to antimicrobial susceptibility test, 2(13.3%) were equally resistant to oxytetracycline, tetracycline, procaine penicillin G and cloxacillin, and all of the isolates (100%) were resistant to sulfamethoxazole and nalidixic acid. Interestingly all *L. monocytogenes* isolates 15 (100%) were sensitive to erythromycin and clindamycin. The detailed patterns of susceptibility presented in Table 5. The study showed the multi-drug resistance pattern of isolates (Figure 7).

Table 5: Antibiogram of *L. monocytogenes* isolates

Antimicrobial agents (symbol)	**Disc content**	**Number of isolates (%)**		
		R	**I**	**S**
Oxytetracycline (OT)	30 μg	2 (13.3%)	2 (13.3%)	11 (73.3%)
Tetracycline (T)	10 μg	2 (13.3%)	10 (66.7%)	3 (20%)
Sulfamethoxazole (RL)	100 μg	15 (100%)	0 (0%)	0 (0%)
Procaine penicillin G (PG)	10 μg	2 (13%)	0 (0%)	13 (87%)
Clindamycin (C)	30 μg	0 (0%)	0 (0%)	15 (100%)
Streptomycine (S)	10 μg	0 (0%)	6 (40%)	9 (60%)
Nalidixic acid (NA)	30 μg	15 (100%)	0 (0%)	0 (0%)
Erythromycin (E)	15 μg	0 (0%)	0 (0%)	15 (100%)
Cloxacillin (OB)	5 μg	2 (13.3%)	2 (13.3%)	11 (73.3%)

R= Resistant S= Sensitive I= Intermidate

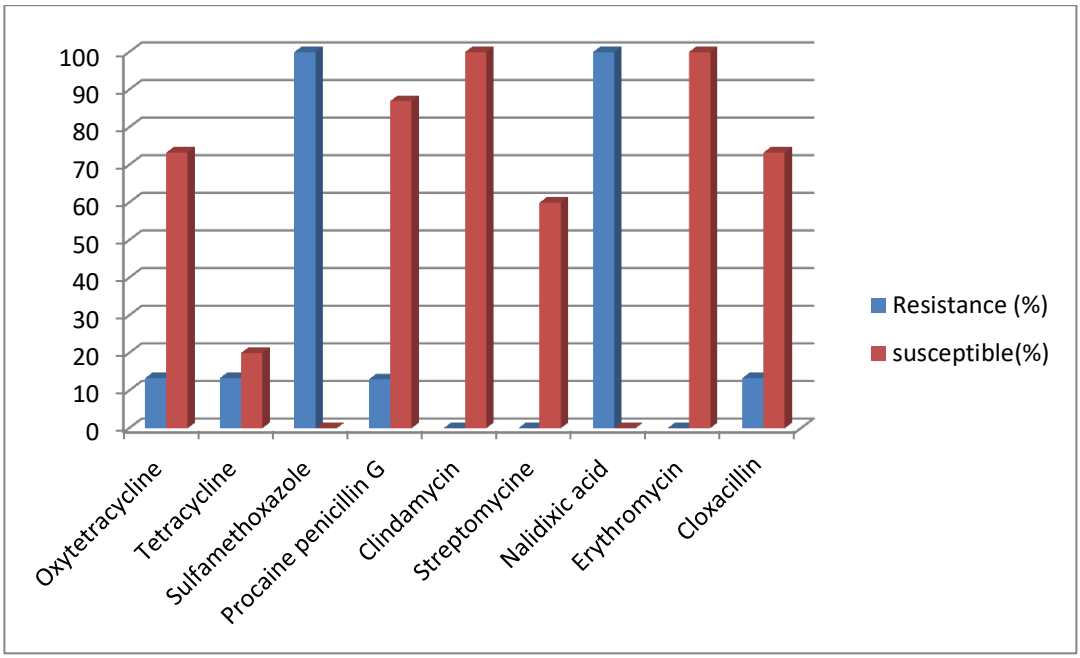


Figure 7: Antimicrobial susceptibility test of *L. monocytogenes* isolates

4.3. Questioner findings

For questioner survey, a total of 70 respondents were used. Out of which 53 were male and 17 of them were female respondents. All of (100%) dairy farmer respondents clean and smoke their milking equipment every day. Only about 3% of the respondents wear protective clothing during milking. Based on questioner findings, 76.7 % of dairy farmers and 20 % of public respondents consume raw milk and 95 % of respondents from the community consume other dairy products including ice cream. About 73.3 % and 12.5 % of farmer and public respondents respectively knew about zoonotic. Based on the observational assessment, 70 % of dairy bedding is dirty and 60 % of milking area and milking equipment were in moderate hygienic status. During the survey, no cow showed any reproductive disease signs and no history of abortion was recorded. Detailed questioner findings are presented in the tables below (Table 6&7).

Table 6: Summary of knowledge, attitude and practices of dairy farmers regarding hygiene and observational assessment of the farm

Variables	Performance	No. of respondents	%	p-value (0.95 CI)
Sex	Male	11	36.7	0.762
	Female	19	63.3	
Educational background	G-10 and above	21	70	0.934
	Lower than G-10	9	30	
Washing hands per day time interval	Once	10	33.3	0.003
	Twice	17	56.7	
	Other	3	10	
Washing of hands	With water only	11	36.7	0.034
	With soap	19	63.3	
Washing of hands after toilet	Yes	7	23.3	0.016
	No	23	76.7	
Consuming raw milk	Yes	23	76.7	0.986
	No	7	23.3	
Cleaning of udder and teat	With water only	18	60	0.019
	With soap	9	30	
	Not clean properly	3	10	
Frequency of cleaning udder area and teat	Every milking	21	70	0.047
	When getting dirt	6	20	
	Not cleaning	3	10	
Clean and smoke milking equipment	Every day	30	100	0.005
	Twice a week	0	0	
	3 times a week	0	0	
Wearing closing during milking	Yes	9	3	0.012
	No	21	97	
View of dairy beading/barn	Clean	3	10	0.003
	Medium	6	20	
	Dirty	21	70	
History of abortion and/reproductive disease	Yes	0	0	0.041
	No	30	100	
Contact with aborted fetus	Yes	0	0	0.058
	No	30	100	

Knowledge of zoonotic disease	Yes No	22 8	73.3 26.7	0.059
Hygiene status of milking area and equipment	Good Moderate Poor	5 18 7	16.66 60 23.33	0.0426
Training on personal and/food hygiene	Yes No	0 30	0 100	0.810

Table 7: Summary of knowledge and attitude of respondents on Hygienic practice, raw milk consumption and zoonotic disease

Variables	**Performance**	**No. of respondents**	**%**
Sex	Male	36	90
	Female	4	10
Educational background	G-10 and above	34	85
	Lower than G-10	6	15
Washing hands per day time interval	Once	3	7.5
	Twice	22	55
	Three times or more	15	37.5
Washing of hands	With water only	2	5
	With soap	38	95
Washing of hands after toilet	Yes	27	67.5
	No	13	32.5
Consuming raw milk	Yes	8	20
	No	32	80
Consumption of other dairy products including ice cream	Yes	38	95
	No	2	5
Knowledge of zoonotic disease	Yes	5	12.5
	No	35	87.5
Live in a farm area	Yes	1	2.5
	No	39	97.5
Training on personal and/food hygiene	Yes	0	0
	No	40	100

5. DISCUSSION

Listeria is an important bacterium that causes a significant disease both in animals and humans. In the majority of human cases, infection is due to the consumption of contaminated foods. Its presence and growth in foods contribute to outbreaks of listeriosis (Jemmi and Stephan, 2006). Studies indicated that *Listeria* species are more prevalent in the milk processing environment (Vitas, 2004).

In this study, raw bovine milk samples were examined for the presence of *L. monocytogenes* and other *Listeria* species. In this finding, the overall prevalence of *Listeria* species was 12.8% (49 out of 384 samples). The high prevalence of *L. monocytogenes* (4.2%) as compared with other *Listeria* species indicated a significant public health hazard.

5.1. Prevalence of *L. monocytogenes* and other *Listeria* species

The prevalence of *Listeria* species in this study is slightly lower than that of the studies reported with a 20.3% prevalence of raw bovine milk from dairy producers in Debre-Berhan town (Girma and Abebe, 2018) and in central highlands of Ethiopia with a 28.4% prevalence from 443 milk and milk product samples (Seyoum *et al*., 2015). This could be due to an increase in hygienic practices as raw milk was considered a source of contamination by dairy farmers, processers and consumers, and sample size differences.

In the current study, the prevalence of *L. monocytogenes* was 4.2%. This is in agreement with Mulu (2014) who noted a prevalence of 4.1% in Addis Ababa, and Mansouri-Najand *et al*. (2015) who noted a prevalence of 5% from raw milk samples in Iran. However, the prevalence of *L. monocytogenes* is reported higher in other countries like in the USA with 26% (John, 2019), and Australia with 40% prevalence (MacGowan *et al*., 1994). The sensitivity of microbial detection methods and sample size differences could partially explain these differences. In Ghana, the low prevalence of 8.8% (Owusu-Kwarteng *et al*., 2018) was

reported. The reason for this is due to different hygienic and sanitary activities in milk supply chains, environmental conditions, and different sample sizes. In Ethiopia, very few studies were done on listeriosis.

A report by (Teshome *et al*., 2019) indicted a prevalence of 5% *L. monocytogenes* in raw milk and milk product samples from Bishoftu and Dukem towns. Abera *et al*. (2007) also noted 4.1% of *L. monocytogenes* from food samples in Addis Ababa. This is also similar to the present study. During this study, isolates of *L. innocua* and *L. monocytogenes* were found to be higher as compared with other *Listeria* species. This is in agreement with the studies by (Molla *et al*., 2004; Gebretsadik *et al*., 2009; Garedew *et al*., 2015; Girma and Abebe, 2018). All reported higher prevalence of *L. monocytogenes* and *L. innocua* comparing with other species.

In Ethiopia, a relatively low prevalence of *L. monocytogenes* from 873 meat swab samples was reported in Addis Ababa at a 4.1% prevalence (Mulu, 2014). This could be due to hygienic conditions, and sample origins. In this study from both districts, the prevalence of *L. monocytogenes* was found slightly higher in Kombolcha town. This could be due to a difference in sampling size. In this finding, the prevalence of *Listeria* species was high in poorly managed and large herd size dairy farms. This might be due to poor hygienic conditions and external contamination via feed, milking equipment, and personnel since the pathogen can multiply and survive up to 7 weeks in dairy manure (Kim & Jiang, 2010). A study which was done by (Vilar *et al*., 2007) also reported a high prevalence of *L. monocytogenes* in reduced ventilation environments as compared to ventilated ones.

5.2. Antibiotic susceptibility profiles

The Antibiogram profile of *L. monocytogenes* was also studied in this work based on the standard Kiby-Bauer disc diffusion method. The results were interpreted based on standards by NCCLS for gram-positive organisms (CLSI, 2004).

In this study, all 15 *L. monocytogenes* isolates were analyzed for antimicrobial susceptibility profile. The result showed that all of the isolates (100%) were resistant to sulfamethoxazole and nalidixic acid and about 13.3% of isolates were resistant to oxytetracycline, tetracycline, procaine penicillin G, and cloxacillin. This is contrary to a report by Garedew *et al*. (2015) who noted 0% of isolates were resistant to sulfamethoxazole. However, this study showed similar findings to Girma and Abebe (2018) who noted 30.5% and 25% of isolates were resistant to nalidixic acid and tetracycline respectively. Another study by Welekidan (2019) stated 66.7% of *L. monocytogenes* isolated from pregnant women were resistant to procaine penicillin G.

This study showed 13.3% of isolates were resistant to oxytetracycline and procaine penicillin G antimicrobial drugs which are commonly prescribed in study areas for the treatment of most bacterial infections. This could be mentioned as one of the reasons for some non-effective treatments with these drugs. The resistance for nalidixic acid and tetracycline was also similar to studies carried in different countries (Pintado *et al*., 2005; Sharma *et al*., 2012). Antimicrobial-resistant *L. monocytogenes* in raw food products have significant public health implications, particularly in developing countries where antibiotic use is prevalent and uncontrolled (Sharma *et al*., 2012). All *L. monocytogenes* isolates were sensitive to clindamycin and erythromycin. This reveled in agreement with studies by Welekidan (2019) and Abera (2007). Both reported 100% of isolates were sensitive to erythromycin. In this study, 87%, 73%, and 60% of isolates were sensitive to procaine penicillin G, oxytetracycline, cloxacillin, and streptomycin respectively. Procaine penicillin G and tetracycline sensitivity were higher than a report by (Mereta *et al*., 2020). The sensitivity of *L. monocytogenes* to streptomycin, procaine penicillin G, tetracycline, and oxytetracycline observed in this study were similar to studies by (Abera, 2007; Mulu, 2014; Garedew *et al*., 2015; Girma and Abebe, 2018).

5.3. Questioner survey

Farmers and personnel can contaminate food products with *L. monocytogenes* if they are not followed proper farm management systems. Poor hygienic practices such as; not wearing personal protective clothing, improper hand washing, not cleaning equipment and working areas properly can result in contamination of foods with *L. monocytogenes* (Cutter *et al*., 2006).

The results of this study showed that most of the respondents (79%) wash their hands daily with soap and 46 % of respondents wash their hands after the toilet. This is in agreement with a previous study which revealed that 80% of respondents use detergent for washing their hands (Mulu, 2014). However, a report by Mulu (2014) showed 94% of respondents wash their hands after using a toilet. Concerning dairy farmers, 60% of the respondents clean the udder area and teat with water only, 30% were using soap to clean and 10 % were not clean properly. This could be a reason for *Listeria* species prevalence in raw bovine milk. Concerning personal and/food hygiene training, this report showed that all respondents were not taking the training yet. This is contrary to previous studies (Mulu, 2014; Mereta *et al*., 2020). This study revealed that consumption of raw milk is higher in dairy farmers which showed a high risk of getting an infection with *L. monocytogenes*. Interestingly, since consumption of raw meat, milk and milk products is very common and a large amount of high-risk population are found the problem can be higher in Ethiopia. In this finding prevalence of *Listeria* was found higher in farms not practicing cleaning of udder and milk equipment. This might be due to the easy transmission and survival of the pathogen from the environment to dairy cows since the origin of *L. monocytogenes* contamination in milk is mainly of faeces (Sauders & Wiedmann, 2007). About 73.3 % of dairy farmer respondents knew about zoonotic disease. This could be due to they learn about those diseases when they got service in veterinary clinics.

6. CONCLUSION AND RECOMMENDATIONS

Generally, the main objectives of this study were the isolation of *L. monocytogenes* and to investigate its prevalence in smallholder dairy farms. *L. monocytogenes* and other *Listeria* species were isolated from raw bovine milk samples. The microbiological laboratory examination of these samples in this study revealed that the significant presence of *L. monocytogenes* comparing with other *Listeria* species. This existence of *L. monocytogenes* in raw milk and its multi-drug resistance pattern is an indication of a serious public health hazard for raw milk and milk product consumers especially to high-risk groups such as; pregnant women, immunocompromised individuals, young and elderly. In this finding dairy farm management practice and herd size of the farm were statistically significant risk factors associated with listeriosis. These factors should be considered a serious hazard and addressed in control measures. Due to the increase of multi-drug resistance showed by *L. monocytogenes* isolates, continuous surveillance of drug resistance is important for effective treatment. The questioner findings in this report showed that most dairy farmers and some public respondents consume raw milk. Associated with the probable risk of getting infected with *Listeria* is higher with increased consumption of raw milk and milk products, this emphasizes the impact of listeriosis in public health and the need for strong control and prevention strategy.

Finally, based on the above findings, the following points are forwarded as recommendations:

- Milk must be boiled enough or subjected to pasteurization before consumption.
- Food safety practices should be implemented starting from production up to consumption with particular emphasis on ready-to-eat foods.
- Good management systems and hygienic measures should be practice in every dairy farm.
- Detail understanding of the source and risk factors of the bacteria is important to create effective control and prevention programs.
- Training and education on food and personal hygiene.

- Creating awareness to farmers and public education about the zoonotic disease in general and their source of infection, mode of transmission, and control measures.
- Regular detection of antibiotic resistance profiles of the pathogen along with considering genetic mechanisms.
- Further researches and extensive surveys on the prevalence of *Listeria monocytogenes* and molecular characterisation of isolates must be undertaken.

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8. APPENDICES

Annexes I: Questionnaire format

Date _____

Code _____

For farmers

Observational assessment and knowledge of farmers and hygienic practices during milking, milk collection, handling, storage, and traditional processing.

1. Farmer's name _____ Sex_____ Address _____ Kebele _____ Educational status_____

2. How many cows do you have? And how many are lactating? Total cows _____ No of milking cows_____

3. How often do you wash your hands/day? Once _____ Twice _____ Other ____

4. Washing hands with water only _____With water and soap ____

5. Do you wash your hands after the toilet? Yes ____ No ____

6. Do you consume milk in raw? Yes _____No_____

7. Do you clean and disinfect Udder area and teats of cow? Clean only with water_____ clean with soap and water_____ clean and disinfect_____

8. Frequency of cleaning udder and teat; every milking____ when getting dirt_____

9. Washing milk collection can: every day _____ smoking_____

10. The view of the dairy bedding? Clean ____ medium ____ dirty_____

11. History of abortion and/reproductive disease? Yes_____ No_____

12. Contact with aborted fetus? Yes____ No_____

13. Knowledge of listeriosis? Yes_____ No_____

14. Knowledge of zoonotic disease other than listeriosis? Yes_____ No_____

15. Clothing used during milking? Yes _____No_____

16. Hair covering during milking; covered_____ not covered _____

17. Hygienic status of the milk collection and storage area and materials? Good _____ Moderate _____ Poor _____

18. Cows feed? Dry hay _____ concentrate_____ grazing ____ mixed_____

19. Training on food and/or personal hygiene? Yes___ No_____

20. Breed of cow; local _____cross with blood level ____

21. Washing equipment's; after work _____ before work_____

For public

1. Respondent's name_____ Sex_____ Address _____Educational status_____

2. Do you consume milk in raw? Yes _____No_____

3. Do you consume yogurt, cheese, ice cream and other milk products? Yes___ No_____

4. Do you wash your hands after toilet? Yes ____ No ____

5. How often do you wash your hands/day? Once _____ Twice _____ Other ____

6. Washing hands with water only _____With water and soap ____

7. Do you take Training on food and/or personal hygiene? Yes___ No_____

8. Do you live in farm area? Yes____ No____

9. Knowledge of zoonotic disease? Yes____ No____

Annex II: sample collection sheet

No	Date	Sample code	Owner name	District	Kebele	Breed	Stock size	Farm Mgt
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								

Annex III: Laboratory data collection sheet

							Carbohydrate utilization testes			
Sample code	Isolation	Latex Agglutination	Gram stain	Motility	Catalase	Hemolysis	D-mannitol	Rhamnose	Xylose	CAMP Test

Annex IV: Antimicrobial susceptibility test result recording sheet

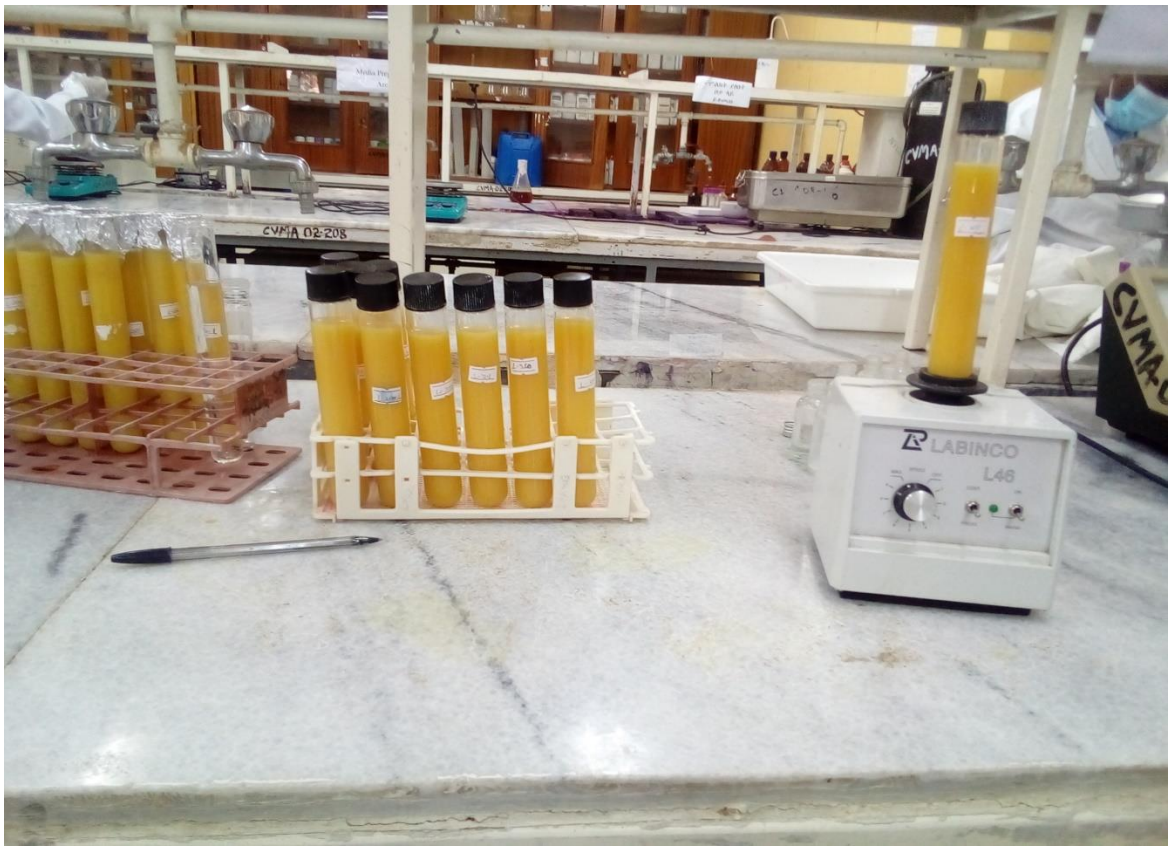
Sample code	antibiotic discs symbols (disc content in µg) and inhibition zone measures in mm								
	OT(30)	T(10)	RL(100)	PG(10)	C(30)	S(10)	NA(30)	E(15)	OB(5)
L-321									
L-323									
L-316									
L-307									
L-252									
L-317									
L-270									
L-133									
L-275									
L-260									
L-314									
L-273									
L-309									

Annex V: Pictures that were taken during the study period

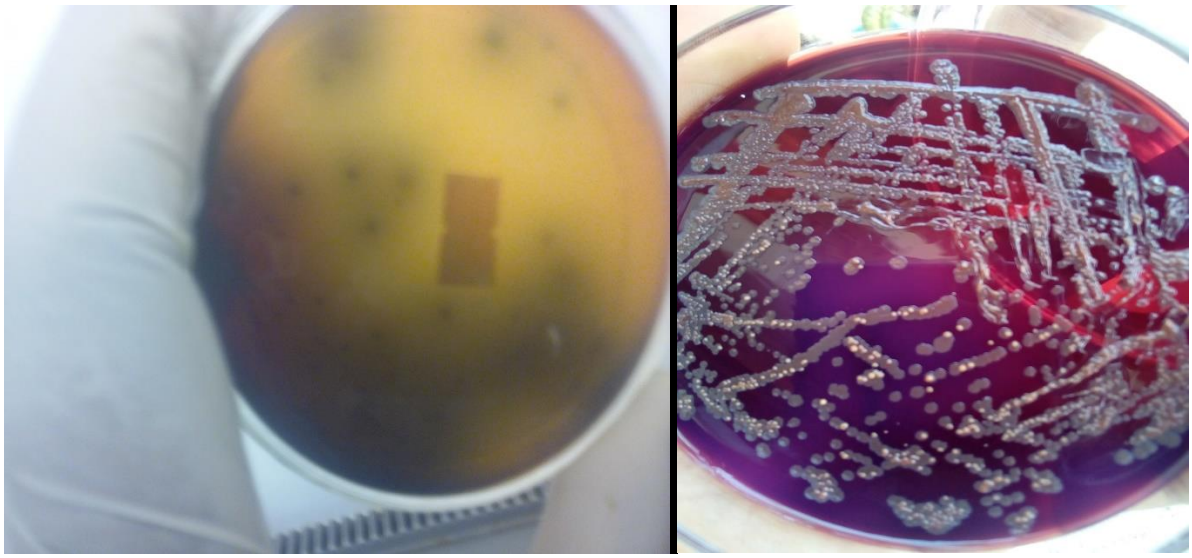
Picture 1: samples in plastic bags mix in primary enrichment with BLEB using laboratory blender (stomacher) in right and selective *Listeria* enrichment supplements in left

Picture 2: Working on microbiological safety cabinet

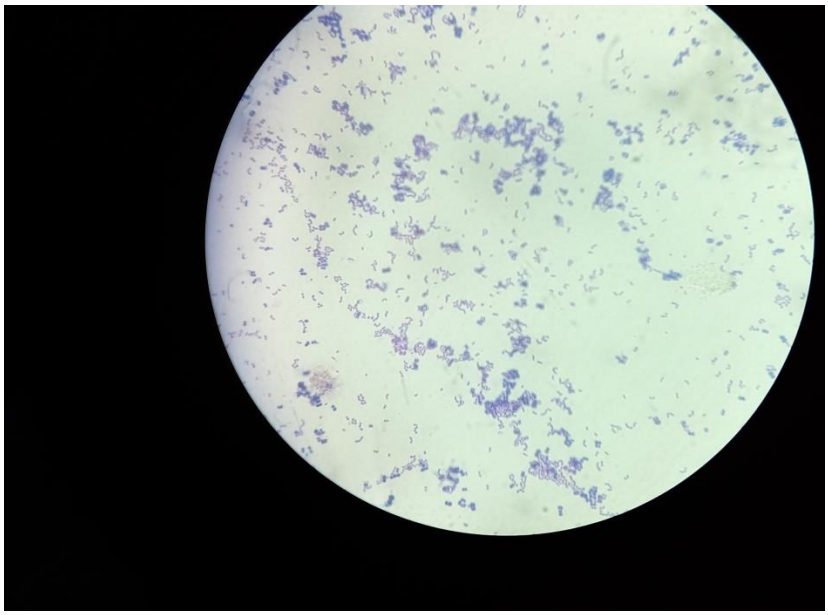
Picture 3: Sample enrichment using test tubes



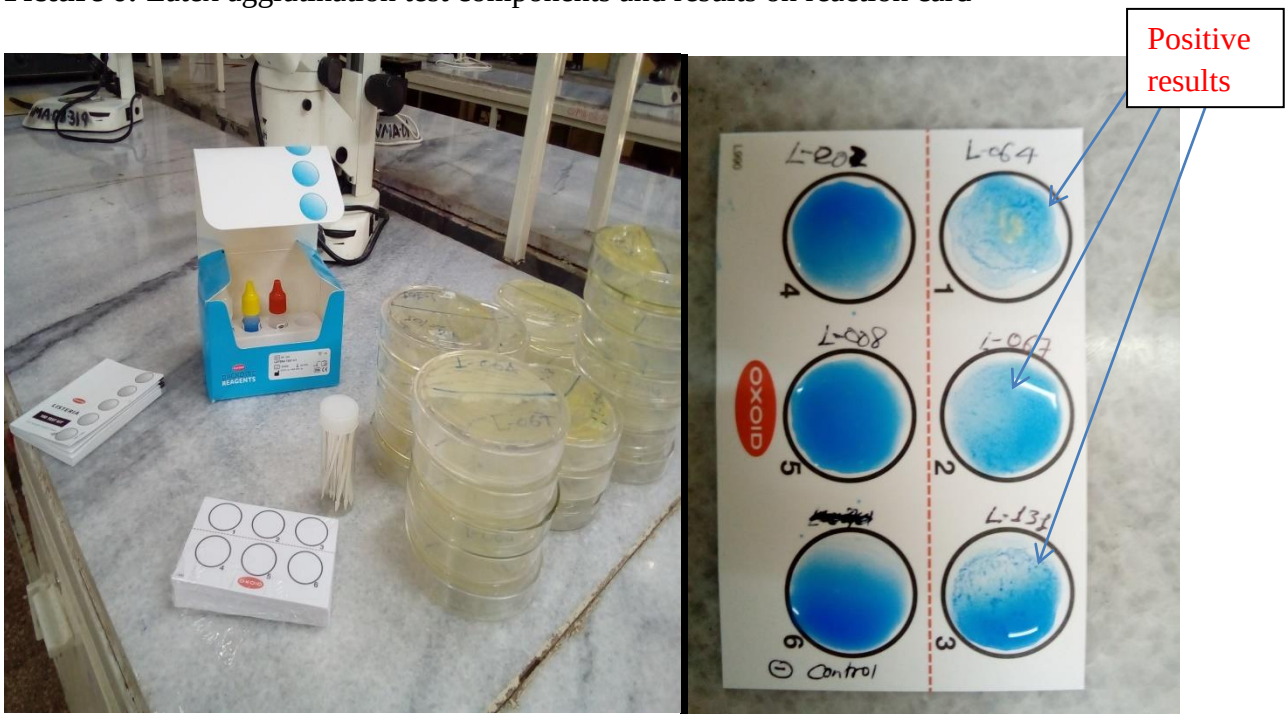
Picture 4: Suspected *Listeria* colonies



Picture 5: Appearance of *Listeria* on gram staining under a microscope

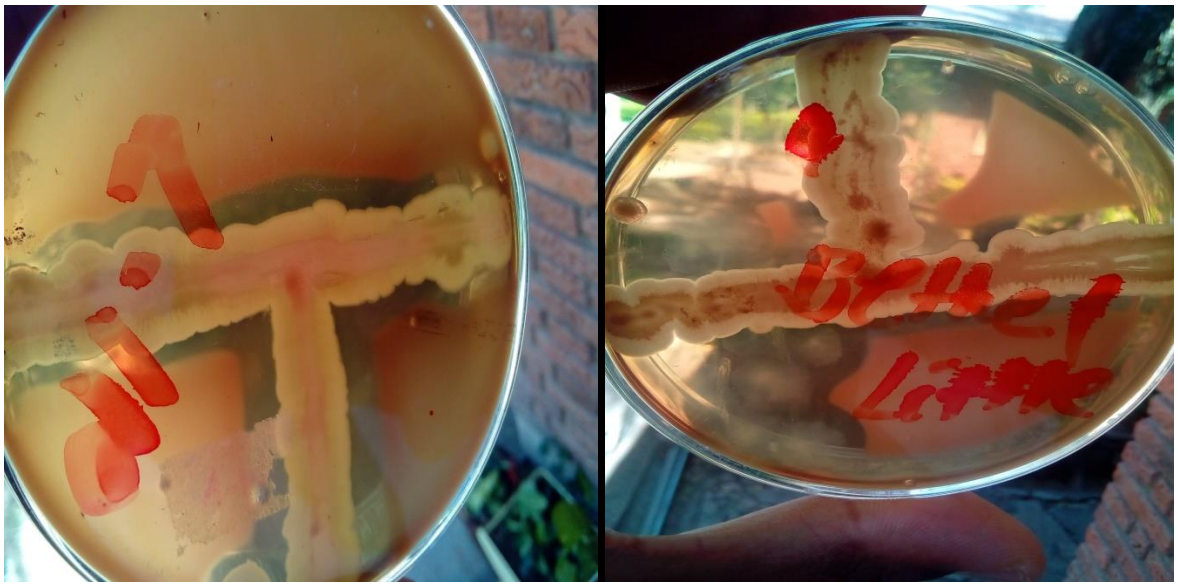


Picture 6: Latex agglutination test components and results on reaction card

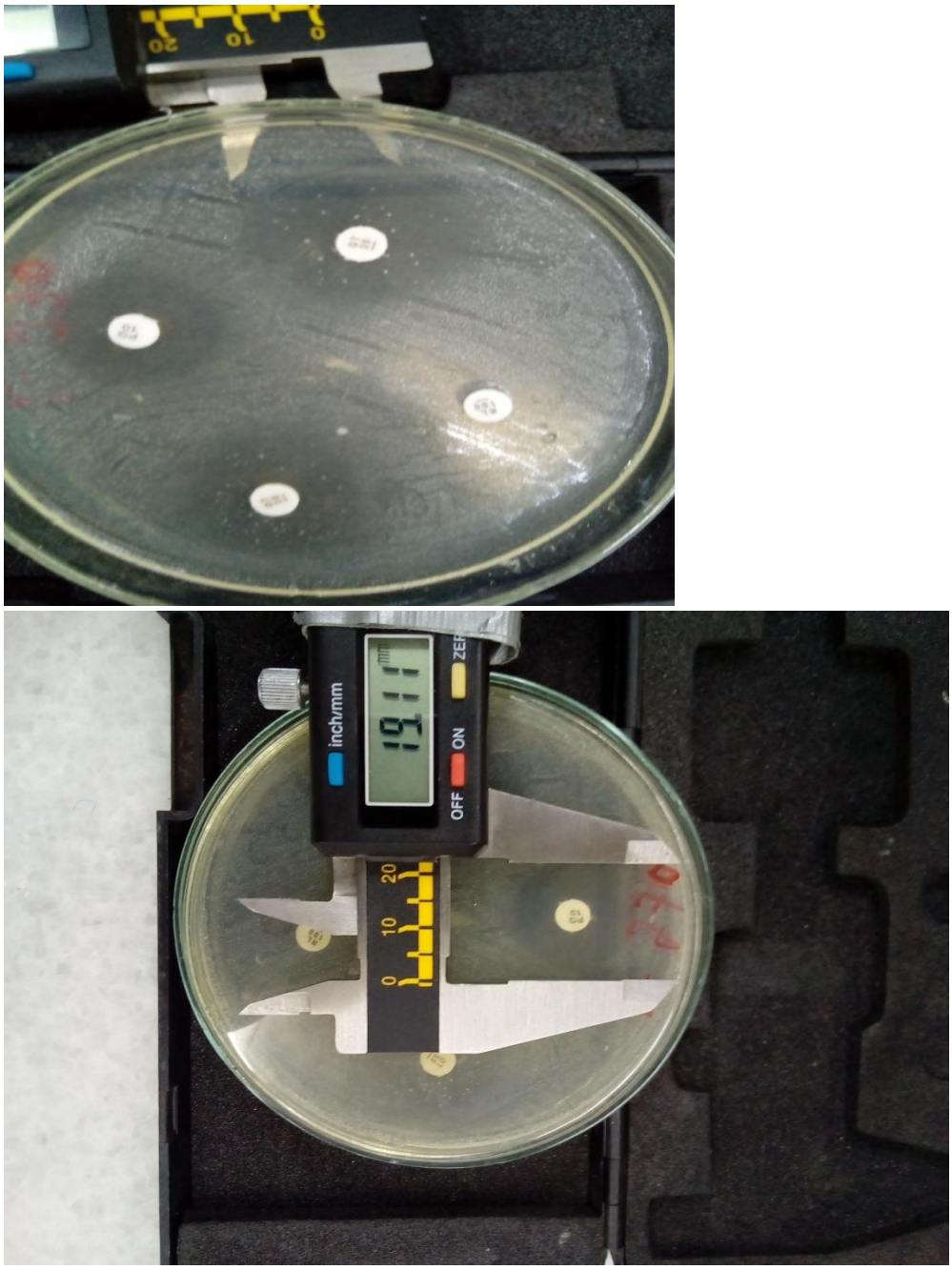


Picture 7: Carbohydrate utilization test; sugars used on right and fermentation results (yellow color) on left

Picture 8: CAMP test positive results



Picture 9: Antimicrobial susceptibility test appearance



Annex VI: Type, principles, procedures, and preparations of Medias used

1. Enrichment

Buffered *Listeria* enrichment broth (BLEB) and Listeria selective enrichment supplement was used

Specifications: CM0897 and SR0141 respectively

Use: A selective enrichment medium for the detection of *Listeria*

Composition; BLEB

Components	**g/L**
Tryptone soya broth	30.0
Yeast extract	6.0
Potassium dihydrogen orthophosphate	1.35
Disodium hydrogen orthophosphate	9.60

Final pH 7.3 ± 0.2 @ 25°C

Composition; *Listeria* selective enrichment supplement

Contents of 1 vial	**/vial**	**/litre**
Nalidixic acid	20.0 mg	40.0 mg
Cycloheximide	25.0 mg	50.0 mg
Acriflavine hydrochloride	7.5 mg	15.0 mg

Preparations:

- ✓ 23.5 g of the powder was added to 500 ml of distilled water and mix well until dissolved and added.

- ✓ Then Sterilize by autoclaving at 121 °C for 15 minutes. Cool down slowly and aseptically pours into sterile plastic bags/test tubes in volumes as required. (Supplement may be added post sterilization).
- ✓ After adding the sample in plastic bags/test tubes contains BLEB incubate it at 30 °C for 4 hours.
- ✓ After 4 hours of incubation aseptically add supplement as the volume required and continue incubation at 30 °C for 48 hours.

2. *Listeria* isolation agar base (PALCAM)

Specification: M1064I

Use: For detection and enumeration of *Listeria* from food and animal feeds.

Composition:

Components in gms/ Liter

Peptone, special……. 23.000
Corn starch……1
Sodium chloride…… 5.000
Yeast extract……. 3.000
D-Mannitol……10.000
Dextrose, anhydrous…… 0.500
Esculin……. 0.800
Ferric ammonium citrate…..0.500
Lithium chloride……..15.000
Phenol red…… 0.080
Agar……. 10.000
Final pH (at 25°C) 7.2±0.2

Preparation:

- ✓ Add 34.44 grams powder in 500 ml distilled water and heat to boiling and dissolve the medium completely.
- ✓ Followed by Sterilize by autoclaving at 15 lbs. pressure (121 °C) for 15 minutes.
- ✓ Then Cool to 45-50 °C and aseptically add rehydrated contents of 1 vial of Supplement (FD061). Mix well and pour into sterile Petri plates.

***Listeria* Selective Supplement (PALCAM)**

Specification: FD061

Use: An antimicrobial supplement used for the selective isolation and identification of *Listeria.*

Composition;

Components Concentration

Polymyxin B sulfate…….. 5000IU

Ceftazidime……. .10mg

Acriflavine hydrochloride…… 2.500mg

3. Nutrient agar

Specification: M001

Use: used for the cultivation of less fastidious microorganisms

Composition;

Components gms / Liter

Peptone ……..5.000

Sodium chloride……. 5.000

HM peptone B#..... 1.500

Yeast extract……. 1.500

Agar…….. 15.000

Final pH (at 25°C) 7.4±0.2

Preparation:

- ✓ 28.0 grams added to 1000 ml distilled water and heat to boiling until dissolve completely.
- ✓ Followed by Sterilization by autoclaving at 121 °C for 15 minutes.
- ✓ Then Cool down and pour into sterile Petri dishes.

4. Nutrient broth

Specification: M002

Use: for the general cultivation of less fastidious microorganisms.

Composition;

Components	**gm /lit**
Peptone……..	5.000
Sodium chloride……	5.000
HM peptone B#.....	1.500
Yeast extract……	1.500

Final pH (at 25 °C) 7.4±0.2

Preparation:

- ✓ 13.0 grams powder was dissolved in 1000 ml distilled water, Heat to dissolve the medium completely.
- ✓ Pour into tubes or flasks as desired then Sterilize by autoclaving at 121 °C for 15 minutes.

5. Blood Agar

Use: for detection of hemolysis

Composition;

Ingredients g/L

Heart infusion from (solids) ……..…2.0

Pancreatic digest of casein……....13.0

Yeast extract ……..…5

Agar……...15.0

Sodium chloride ……5.0

Preparation

- ✓ 40 gram of powder dissolved in 1liter of distilled water and boil until dissolved completely.
- ✓ Sterilized by Autoclaving at 121^0 C for 15 minutes.
- ✓ Then cool to 45-50 ^{o} C and add 5% sterile, defibrinated sheep blood. Finally, Dispense into sterile Petri-dishes.

6. Muller- Hinton Agar

Specification: MM019

Use: for antibiotic susceptibility test of bacterial isolates

Composition:

Ingredients gm/lit

Beef infusion from……300.0

Acid hydrolysate of casein……17.50

Agar……...17.00

Starch……1.5

pH at 25 ^{o}C : 7.4 ± 0.2

Storage: Store between 8-25 ^{o}C

Preparation:

- ✓ Add 39.0 gm of powder dissolved in 1.0 liter distilled water, boil until dissolve completely.
- ✓ Autoclave at at 121 ^{o}C for 15 minutes.
- ✓ Pour into sterile Petri dish

7. Carbohydrate utilization test

Sugar: D (+)-Mannose**;** D-Mannopyranose

Lot No: 122K0016

25 g

Product: Sigma-Aldrich, Steinheim, Germany 49-7329-970

Sugar: D (+) –Xylose 99%

Lot No: S14974-513

100 g

Product: Sigma-Aldrich, Steinheim, Germany

Sugar: L (+) – Rhamnose-Monohydrat

25 g

Product: E Merck 64271, Darmstadt, Germany

Phenol red starch broth

500 g Lot 108H0985

Usage: 21 g/L

Made in: Sigma-Aldrich, Steinheim, Germany

8. Oxoid *Listeria* test kit

Specification: DR1126A

Product of Oxoid, Basingstoke, Hampshire, England

Kit components;

- *Listeria* latex reagent: Latex particles coated with rabbit antiserum against *Listeria* flagellin antigens, preserved with 0.02% Merthiolate. 1*5ml
- *Listeria* positive control antigen 0.5ml
- 0.85% isotonic saline, preserved with 0.09%sodium azide 5ml
- Disposable reaction cards

Controls

a. Reagent control

- ✓ Add 1 drop of Oxoid *Listeria* latex reagent to 1 drop of saline in the same circle on the reaction card.
- ✓ Mix and observe for agglutination for 2 minutes.
- ✓ No agglutination should occur. If this control shows agglutination either the latex regent or the saline is probably contaminated and should be discarded.

b. Positive control

- ✓ Place a drop of positive control antigen suspension on a reaction card.
- ✓ Add 1 drop of latex reagent in the same circle next to the drop of positive control suspension. DO NOT ALLOW THE DROPPER TO TOUCH THE POSITIVE CONTROL.
- ✓ Mix the latex reagent and the positive control with a clean mixing stick spreading the suspension over the entire surface of the circle. Gently rock and examine for agglutination within a maximum of 2 minutes. Easily discernable agglutination with the latex reagent indicates normal reagent function.

Test procedure

- ✓ Place a reaction card on the workbench
- ✓ Add 1 drop of saline within one circle on the reaction card
- ✓ Using a mixing stick or inoculating loop emulsify the suspect colony in the drop of saline o produce a heavy smooth suspension
- ✓ Gently mix the Oxoid Listeria latex reagent by inverting and place a drop of reagent in the same well adjacent to the suspension. DO NOT ALLOW THE DROPPER TO TOUCH THE ORGANISM SUSPENSION. Mix the latex reagent and the organism suspension with the clean mixing sick, spreading the mixture over the entire area of the well, and gently rock the reaction card.
- ✓ examine for agglutination for a maximum of 2 minutes
- ✓ After reading, discard the used reaction cards into a suitable disinfectant.

Annex VII: Kirby Bauer disk diffusion method antibiotic susceptibility test interpretation standards (CLSI, 2004).

Antibiotic	**Disc content (µg)**	**Inhibition zone (mm)**		
		S	I	R
Chloramphenicol	30	≥21	18-20	≤ 18
Cloxacillin	5	≥14	10-13	≤ 9
Trimethoprim sulfamethoxazole	25	≥ 16	11-15	≤ 10
Erythromycin	15	≥ 23	14-22	≤ 13
Pencillin G	10	≥34	31-33	≤ 31
Kanamycin	30	≥ 18	14-17	≤ 13
Rifampicin	5	≥ 20	17-19	≤ 16
Streptomycin	10	≥ 15	12-14	≤ 11
Tetracycline	30	≥19	15-18	≤ 14
Vancomycin	30	≥ 12	10-11	≤ 9
Gentamicin	10	≥ 15	13-14	≤ 12
Clindamycin	2	≥ 18	14-17	≤ 13

Annex VIII: Ethical clearance certification sheet

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/19/5/13/2021

Name of Applicant: **Yitbarek Getachew (DVM, PhD)**

Address: Department of Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Improving dairy cattle productivity: unraveling the epidemiology of emerging and reemerging infectious diseases of reproduction and feed related metabolic disorders towards developing efficient intervention strategies to ensure food security and public safety*

Date of application:	**February, 2021**
Nature of the project:	**Mildly invasive**
Target animal species:	**Cattle**
Number of animals involved:	**1500**
Study area:	**Different parts of Ethiopia**

Minutes No. and date of review: **VM/ERC/05/13/021, 21/03/2021**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

Getachew Terefe (DVM, PhD, Professor of Vet. Parasitology)
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine and Agriculture

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Bishoftu, Ethiopia

Annex IX: Plagiarism result

Curiginal

Document Information

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