ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

STUDY ON PREVALENCE OF LISTERIA SPECIES IN RAW MILK AND COTTAGE CHEESE IN DEBRE ZEIT, ETHIOPIA.

BY
ABDUL JELAL REDI

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A Thesis Submitted to the Faculty of Veterinary medicine, Addis Ababa University In Partial Fulfillment of the Requirements for the Degree of Master of Tropical Veterinary Public Health

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ACADEMIC ADVISOR:

DR MOSES KYULE (BVM, Msc, MVPM, PHD)

ACKNOWLEDGEMENTS

I am greatly indebted to my advisor Dr. M. Kyule for his advice and correction of the paper. He was always following my work in the laboratory throughout my study period. He deserves a lot of acknowledgements since he thought me the way how to prepare the paper and analyze the data and this is a good opportunity for me to express my great respect and appreciation to him.

I am also grateful to Dr. Girma Zewde, head of the department of Micro biology and Public Health for regular visits in the laboratory and for his advice.

I am also grateful to my colleagues Dr. Mekonen Addis, Dr. Assaminew Tesfaye, Dr. Rediate Tesfaye and externship students Fisseha and Biruke for the times we spent in the lab.

Ato Anteneh and Hirut are also greatly acknowledged for their unreserved effort in making working materials ready for use in our daily activities.

I would like to extend my appreciation and word of thanks to workers of Ada Dairy co-operative for there UN reserved effort during sampling.

Using this opportunity, I would like to express my heartfelt thanks to Cheha Woreda Agricultural Office for giving me the opportunity to attend postgraduate study and for securing my monthly Salary throughout my study period.

It is my pleasure to express my great love and respect to all of my family members.

TABLEOF CONTENTS **Page**

LIST OF TABLES **Page**

LIST OF FIGURES page

LIST OF APPENDICES

page

ABBREVATIONS

AIDS	Acquired Immuno Deficiency Syndrome
ARDS	Adult Respiratory Distress Syndrome
CAMP	Christie, Atkins, Munch-Petersen
CDC	Centers for Disease Control
CFU	Colony forming unit
CNS	Central Nervous System
CSA	Central statistical Authority
DIC	Disseminate Intravascular Coagulation
DNA	Deoxyribo Nucleic Acid
FDA	Food and drug administration
HACCP	Hazard analysis critical control point
HIV	Human Immunodeficiency Virus
INA	Internaline A
INlB	Internaline B
ISO	International Organization for Standardization
Lab	Laboratory
LLO	Listeriolysine O
MHA	Muller Hinton agar
MID	Minimum infectious dose
Ms-Excell	Microsoft excell
RNA	Ribo Nucleic Acid
RTE	Ready to eat
SIM	Hydrogen Sulphide, Indole and Motility Medium
SPSS	Soft ware program for social science
USDA	United states department of agriculture
WHO	World Health Organization

ABSTRACT

A cross-sectional study of *Listeria* species was conducted on bulk raw milk, farm raw milk and cottage cheese. A total of 400 samples consisting of 100 farm raw milk, 100 bulk raw milk and 200 cottage cheese samples were collected. Out of these samples, 49 (12.25%) were positive for *Listeria*. The bulk raw milk was the most contaminated with *Listeria* species with an overall prevalence of 27%. Sixteen samples (16%) of farm raw milk, 6 (3%) of cottage cheese were positive for *Listeria*. *Listeria innocua* was the predominant species (3.75%) isolated. Its prevalence was higher in bulk raw milk with a prevalence of 8% followed by 4% of farm raw milk and 1.5% of cottage cheese. *Listeria monocytogenes* was the second prevalent *Listeria* species isolated (3.5%).The prevalence of *L. monocytogenes* in bulk raw milk was high with a prevalence of 7% followed by farm raw milk (5%) and cottage cheese (1%). Other *Listeria* species isolated were *L. ivanovii* (2%), *L. welshimeri* (1%), *L. seeligeri* (1%), and *L. grayi* (1%). In conclusion, *Listeria* species were found in the different food items examined. This indicated poor hygienic procedures at the farm level and dairy processing plant that led to the contamination of raw milk and cottage cheese products. Since the food items were of ready-to-eat there was a possible risk of food borne listeriosis. Therefore hygienic Monitoring programme should put in place to the critical points with ultimate objectives of producing safe and suitable milk and milk products. The responsible veterinary and public health authorities should make efforts to raise the awareness of food producers, suppliers and retailers about the methods of safe food production, storage and handling and should be monitored and inspected regularly.

Key words: Bulk raw milk, Cottage cheese, Debre Zeit, Farm raw milk, *Listeria* species, and prevalence.

1. INTRODUCTION

Food-borne diseases are one of the major public health problems all over the world. Among the various pathogenic bacteria that are known to cause food borne diseases, the most important are members of the genus *Listeria* (Cordano and Rocourt, 2001). It was first described in 1926 in Cambridge that caused an outbreak in guinea pigs and rodents (Rocourt, 1996). The bacteria in the genus *Listeria* are ubiquitous in nature and have world wide distribution (Al-Dughaym *et al.*, 2001). They are found in decaying vegetation, soils, animal feces, sewage, silage, and water. The bacterium often passes through an animal's and human's intestinal tract without causing illness (Norrung, 2000).

Listeriosis is an infectious disease affecting human, a wide variety of animal and avian species, as well as insect crustaceans and fish acquired mainly through ingestion of contaminated feed/food. The bacteria can grow over a temperature range of about 1°C to 45° C and a pH range of 4.1 to 9.6. It survives in foods for long periods of time (Jay, 2000). It is found in a variety of food and food products including raw milk, soft cheeses, fresh and frozen meat, poultry, seafood products, fruits and vegetables. The highest risk foods include ready-to-eat and refrigerated foods (WHO, 1988).

Listeria monocytogenes has been incriminated in about 98% of human listeriosis, in addition human cases have been known to be caused by *L. ivanovii* and by *L. seeligeri* (Jemmi and Stephan, 2006). Immuno competent people rarely contract this disease, but it can be severe for certain groups of people (especially the elderly, newborn, pregnant women and those with a weakened immune system). *Listeria monocytogenes* can cause abortion as well as meningitis and septicemia in newborn infants, in pregnant women, elderly and immunocompromized people; the case-fatality rate can reach 30 % (Low and Donachie, 1997).

The first food borne outbreak of listeriosis was reported in 1981 in Nova Scotia, Canada (Swaminathan and Smidt, 2007) and 1985 in California that caused 142 cases with 48 deaths was the final alert to the role of food in disseminating listeriosis (Jemmi and Stephan, 2006). Thus listeriosis has emerged as a typical food-borne illness of major public health concern because of its severity (Jay, 2000). Entry of *L. monocytogenes* into the food processing plants is from animals, possibly from healthy human carriers and equipments used for processing (Rocourt and Cossart, 1997).

Milk and milk products have been implicated in the transmission of *Listeria* to humans. Since proper pasteurization kills this pathogen, most milk-borne listeriosis is associated with raw and inadequately pasteurized milk or milk contaminated after pasteurization. Contamination of milk by *Listeria* may occur from the excretion of symptom less or diseased animals, contaminated equipment, floors and also personnel involved in food production chain. Despite increasing preventive measures such as the implementation of the Hazard Analysis Critical Control Point system, the overall incidence of listeriosis has not decreased. In fact, it appears that changes in diet and tendencies for more centralized large-scale food processing have increased the opportunities for transmission of food-borne listeriosis. In order to avoid any major risk, many countries now impose regulations that require producers, processors and distributors of foodstuffs to set up more frequent and efficient testing plans for the systematic control of risk products. Hence, it is appropriate again to indicate that dairy industry personnel, public health and regulatory officials, food microbiologists, and academicians should have a ready source of information regarding the problem. Therefore, periodic surveillance of levels of *Listeria* contamination in the different food animals, food products and environment is necessary to prevent the spread of this pathogen and infection of humans (Akpolat *et al*; 2004).

Two Studies conducted in Ethiopia have revealed the occurrence of *Listeria* species in retail foods in Addis Ababa (Molla *et al*., 2004; Mengesha, 2005). Studies should be designed to establish prevalence of *Listeria* species in different parts of Ethiopia. The recent study was undertaken with the following objectives:

- To determine the prevalence of *Listeria* species in raw milk and cottage cheese in Debre Zeit.
- To compare the prevalence of *Listeria* species in farm raw and bulk raw milk in Ada Dairy Co-operative in Debre Zeit.

2. LITRATURE REVIEW

2.1. Historical perspectives

Listeria monocytogenes (originally named *Bacterium monocytogenes*) is a Gram-positive bacterium first described in 1926 in Cambridge, United Kingdom, as a cause of infection with monocytosis in laboratory guinea pigs and rodents. In the following year, Pirie also isolated a Gram-positive bacterium, in this instance from infected wild gerbils in South Africa, and proposed the name *Listerella* for the genus in honor of the surgeon Lord Lister. Murray and Pirie realized that they were dealing with the same species of bacteria and thus combined the names to form *Listerella monocytogenes*. This was later changed for taxonomic reasons to *Listeria monocytogenes* (Todar, 2008).

2.2. Taxonomy and nomenclature

The Scientific_classification includes- Kigdom: Monera; Division: Firmicutes; Class: Bacilli; Order: Bacillales; Family: Listeriaceae; Genus: *Listeria* (Barichd, 2006). *Listeria moncytogenes* was the only original species described in the genus by Murry (1926) and Pirie (1927). Although originally described as monotypic containing only *L. monocytogenes* now comprise six species which includes: *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, and *L. gray* (Table 1). All of these species are widespread in the environment, but only *L. monocytogenes* and *L. ivanovii* are considered to be a significant pathogen for human and animal (Jemmi and Stephan, 2006). A seventh species (*Listeria murrayi*) was previously recognized within the genus; however, genetic analysis led to the classification of this as a subspecies within *L. grayi.*

Table 1: Classification of the genus *Listeria*

Species name	Reason for name	Date of classification
L. monocytogenes	Infection results in increased monocyte production	1926
L. grayi	Named after M.L. Gray (American bacteriologist)	1966
L. innocua	Infection is harmless	1979
L. welshimeri	Named after H.J. Welshimer (American bacteriologist)	1983
L. seeligeri	Named after H.P. Seeliger (German bacteriologist)	1983
L. ivanovii	Named after I. Ivanov (Bulgarian microbiologist)	1984

Source: Swaminathan and Smidt (2007).

2.3. Characteristics of *Listeria*

Listeria species are Gram positive; non spore forming; non-acid fast; facultative intra cellular (AlDughaym *et al*., 2001). The organism is1-2μm in length 0.5 μm wide (Anon, 2003) with the bacteria often lying parallel to each other, and thread like forms present in some cultures. *Listeria* colonies form a blue green sheen when viewed by obliquely transmitted light. The colonies are small, smooth, slightly flattened and milky white by reflected light (Low and Donachie, 1997; Jay, 2000). Growth occurs between 1°C and 45°C but the optimum temperature range is between 30°C- 37°C (Todar, 2008). The organisms multiply rapidly in anaerobic condition or a micro aerophlic condition at a PH value 4.1- 9.6 (Patricia *et al.,* 2007). Growth is scant or absent in complete anaerobic conditions and multiplication is inhibited `by pH values lower than 4.1. An organism grown at 37°C shows little or no motility but characteristic tumbling motility can be demonstrated by incubation of cultures at room temperature with motile organisms commonly possessing peritrichous flagella.

2.4. Species identification

The identification of *Listeria* species is based on a limited number of bio chemical tests among which carbohydrate utilization and hemolysis are the major differential characteristic.

2.4.1. Carbohydrate utilization test

The carbohydrate utilization tests useful for discriminating between the species are acid production from xylose, rhamnose, and mannitol. The scheme for biochemical identification of *Listeria* species is shown in Table 2.

2.4.2. Hemolysis

Only three species, *L. monocytogenes*, *L. ivanovii*, and *L. seligeri* are hemolytic. They lyse red blood cells from most mammalian animals. The hemolysing activity is most regularly demonstrated using horse or sheep blood-containing agar plates. *Listeria ivanovii* exhibits a wide zone of hemolysis, sometimes even multiple zones. Hemolysis of *L. monocytogenes* resembles that of *Streptococcus agalactiae* (group B streptococci): the zone of hemolysis is narrow, frequently not extending much beyond the edge of the colonies. *Listeria seeligeri* produces even narrower zones of hemolysis. In order to improve the assessment of hemolysis, various authors recommend the use of the CAMP test. The CAMP test, a method widely used for identifying *S. agalactiae*, it uses a β-hemolytic- *Staphylococcus aureus* and a *Rhodococcus equi* strain streaked in one direction on a sheep blood agar plate and test cultures of *Listeria* species streaked at right angles to (but not touching) the *S. aureus* and *R. equi* lines (Allerberger, 2003; Gasanov *et al*, 2005). Hemolysis is essential in differentiating between *L. monocytogenes* and *L. innocua*; the most frequently isolated non-pathogenic *Listeria* species (Gasanov *et al.,* 2005; Jemmi and Stephan, 2006).

Table 2: Carbohydrate utilization by *Listeria* species

Species	Haemolysis	Production of acid			CAMP test	
		Rhamnose	Xylose	manitol	*S. aureus*	*R. equi*
L. monocytogenes	+	+	-	-	+	-
L. innocua	-	V	-	-	-	-
L. ivanovii	++	-	+	-	-	+
L. seeligeri	(+)	+	+	-	(+)	-
L. welshimeri	-	V	+	-	-	-
L. grayi	-	V	-	+	-	-

V: variable; (+): weak reaction; +: positive reactions; -: no reaction

Source: Gasanov *et al*. (2005)

2. 4.3. Serotyping

Listeria species possess surface markers such as somatic (O) and flagellar (H) antigens that demonstrate group specificity and can be utilized as targets for immunological identification and serotyping with corresponding antibodies. *Listeria* somatic (O) antigens have been separated into 15 subtypes (I–XV), and flagellar (H) antigens into four subtypes (A–D). The serotypes of individual *Listeria* strains are ascertained through their unique combinations of O and H antigens (Norrung, 2000). Upon analysis of group-specific *Listeria* O and H antigens via slide agglutination, at least 13 serotypes (i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) are recognized in *L. monocytogenes,* several (e.g., 1/2a, 1/2b, 3b, 4a, 4b, 4c, and 6b) in *L. seeligeri*, one (i.e., 5) in *L. ivanovii*, and a few (e.g., 1/2b, 6a, and 6b) in *L. innocua, L. welshimeri,* and *L. grayi* (Table 3). The H-antigens are useful for identification of *Listeria* species because the antisera generated against them do not cross-react with other bacterial species (Patricia *et al.*, 2007). Using various genetic subtyping techniques, *L. monocytogenes* is separated into three lineages: Lineage I contains serovars 1/2b, 3b, 4b, 4d, and 4e; lineage II contains serovars 1/2a, 1/2c, 3a, and 3c; and lineage III contains serovars 4a and 4c. Strains of *L. monocytogenes* within serovars 4b, 1/2a, 1/2b, and 1/2c account for more than 98% of isolations from clinical cases of human listeriosis, and serovar 4b has been associated with the most recent large outbreaks of listerisois (Dongyou, 2008).

Table 3: Serotypes of *Listeria* species

Species	Serovar designition	O- Antigen	H- Antigen
L. monocytogenes	1/2a	I II (III)	AB
	1/2b	I II (III)	ABC
	1/2c	I II (III)	BD
	3a	II (III) IV	AB
	3b	II (III) IV (XII)(XIII)	ABC
	3c	II (III) IV (XII) (XIII)	BD
	4a	(III) (V) VII IX	ABC
	4ab	(III) V VI VII IX X	ABC
	4b	(III) V VI	ABC
	4c	(III) V VII	ABC
	4d	(III) (V) VI VIII	ABC
	4e	(III)V VI (VIII) (IX)	ABC
	7	(III) XII XIII	ABC
L. ivanovi	5	(III) (V) VI VIII X	ABC
L. innocua	6a	(III) V (VI) (VII) (IX) XV	ABC
	6b	(III) (V) (VI) (VII) IX X XI	ABC
L. grayi		(III) XII XIV	E

Source: Gasanov *et al*. (2005)

2.5. Epidemiology

2.5.1. Distribution

Listeria is world wide in distribution and has long been associated with an animal disease, particularly in herd animals. It is found naturally in soil, decaying vegetation, and in the gastrointestinal flora of most mammals (CDC, 2008). The vagina, cervix, and pharynx are other sites for potential carriage of the organism. *Listeria monocytogenes* has been isolated from soft cheeses, raw milk, raw vegetables, and meats (including fish, poultry, and beef) that are fresh or processed (CDC, 2007). Most cases of human Listeriosis are sporadic in nature, but there have been several reports implicating a common food source outbreak. There have been no documented cases of human-to-human transmission of *Listeria*, although there have been clusters of infections in veterinarians and those who come in close contact with animals (Swaminathan and Smidt, 2007).

2.5.2. Source of infection

Infection in humans occurs mainly via food. It is found extensively in the environment and in the human gastrointestinal tracts. Thus food producers should strive for levels of *Listeria monocytogenes* in their products that are acceptable by the high-risk groups.

The three main factors to be considered in food contamination are (Anon, 1991):

1. The initial source of the bacteria.
2. The ability of the food to support the bacteria.
3 .Post-processing contamination of food

Milk

Milk has also long been associated with *Listeria* contamination. This is because *Listeria monocytogenes* grows well in milk. The source of contamination is normally the animal that produces the milk. It can be contaminated by environmental sources including cow dung, soil, straw and, rarely, by mastitis (Rocourt and Bille, 1997). *Listeria monocytogenes* populations in bulk tank raw milk are usually low (<1 to 10 cfu/ml) (Rocourt and Cossart, 1997). Cows suffering from mastitis caused by *L. monocytogenes* are very rare but in such cases up to 103 cfu/ml may be excreted (Rocourt and Bille, 1997). Raw goat and ewe milk are frequently used for cheese production but there are only limited data on the occurrence of *L. monocytogenes* in these types of milk (WHO, 1988). The process of milk harvesting is probably one of the most important steps in reducing the contamination risk of this product (Schukken *et al.*, 2003). Pasteurization is confirmed to be a safe process, which reduces the number of *L. monocytogenes* occurring in raw milk to levels that do not pose an appreciable risk to human health (WHO, 1988), however, post pasteurization contamination in processing plants has been documented (Schukken *et al.*, 2003). So efforts to ensure that milk is safe from *L. monocytogenes* contamination should focus on identifying and eliminating sources of post-pasteurization contamination (Rocourt and Bille, 1997).

Cheese

Of all foods, cheeses have been found to be frequently contaminated with *L. monocytogenes* and associated with human disease listeriosis suggesting this type of food to be particularly suitable as a vehicle for infection by *L. monocytogenes* (WHO, 1988; Axelsson and Sorin, 1998). Most surveys suggest that 1% to 15% of cheeses are contaminated with *L. monocytogenes* (Rocourt and Bille, 1997). Soft ripened cheeses appear to be most suitable both to contamination and growth of *L. monocytogenes*. This may be due to the higher pH of these cheeses in the later stages of ripening (WHO, 1988). Studies on the behavior of *L. monocytogenes* in various artificially contaminated cheeses showed that substantial numbers of *L. monocytogenes* cells survive the manufacture and ripening of various types of cheeses including cottage cheese.

There is a highly significant correlation between *Listeria* growth and cheese pH values greater than 5.5 and the absence of starter cultures during manufacturing. Variations in manufacturing practices result in opportunities for post-process contamination (WHO, 1988). There are two main ways for *L. monocytogenes* to find its way to cheeses. This pathogen might be present in the raw milk delivered to the dairy or it might also be a part of the resident bacterial flora of the processing plant or ripening room of the dairy and thus constitute a risk of contamination for curd and cheeses. The organism might originally have been introduced not necessarily by means of contaminated raw milk but also by soil, water or dust (Eilertz *et al.*, 1993). Cloet *et al.* (1989) traced the source of *L. monocytogenes* following four occurrences of contamination of cheese with this bacterium through the bulk milk tank to a particular farm, and eventually to a particular cow which was found to be excreting the organism in its milk from one quarter. The organism was also isolated from the feces of the same animal. Certainly, cheese made from UN pasteurized milk has a much higher risk of being contaminated with *Listeria*. WHO has concluded that properly executed pasteurization reduces *Listeria monocytogenes* in milk to levels that "do not pose an appreciable risk to human health" If this is true, it is reasonable to suggest that outbreaks of listeriosis attributed to pasteurised milk and cheeses are due to post-pasteurization contamination (Lunden *et al.*, 2004).

2. 5.3. Outbreaks of listeriosis

The first food-borne outbreak was observed in 1981 in Nova Scotia (Canada) and involved 41 patients. The epidemiological and microbiological investigations suggested that it was caused by a contaminated batch of coleslaw. The next outbreak occurred in Massachusetts in 1983 that caused 49 cases in two months. Based on the case-control study, pasteurized milk was incriminated (post pasteurization contamination is one possible explanation). In 1985 in California, an epidemic of 142 cases was traced to a Mexican-type cheese. Thus, North America experienced three major outbreaks from 1981 to 1985. Since 1983, outbreaks have continued to occur (Jemmi and Stephan, 2006). Contaminated soft cheese was responsible for a four-year outbreak of 122 cases from 1983 to 1987 in Switzerland and a pate caused a 366 patient epidemic in the UK in 1987-1989 (Swaminathan and Smidt, 2007).

In France, a contaminated pork tongue was the source of 279 cases in ten months in 1992 and Rillettes was associated with 38 cases in 1993 (Table 4).

Two studies conducted in Addis Ababa, Ethiopia indicated the presence of *Listeria* species in different foodstuffs. Three hundred and sixteen food samples collected and analyzed in Addis Ababa have indicated 32.6% positive, pork (69.8%) showed the highest prevalence followed by 47.5% in minced beef and 43.5% in ice cream (Molla *et al*., 2004). In another study conducted in Addis Ababa, from 711 samples examined189 (26.6%) were positive, with the highest prevalence of 62.5% in pork followed by minced beef, ice cream and cheese (Mengesha, 2005).

Table 4: International food borne disease outbreaks of invasive Listeriosis, 1980-2003

Year	Location	No. of cases	Perinatal cases	No. of deaths	Suspect/implicated vehicle	Serotype
1981	Nova Scotia, Canada	41	34	18	Coleslaw	4b
1983	Massachusetts, USA	49	7	14	Pasteurized milk	4b
1985	California, USA	142	94	48	Mexican-style cheese	4b
1983-1987	Switzerland	122	65	34	cheese	4b
1987-1989	United Kingdom	366	18	6	Pate	4b
1989-1990	Denmark	26	3	7	Blue mold cheese	4b
1992	France	279	0	85	Pork tongue in jelly	4b
1993	France	38	31	10	Rillettes	4b
1998-1999	Multiple states, USA	108	?	14	Hot dogs	4b
1999	Finland	25	0	6	Butter	3a
1999-2000	France	32	9	10	Pork tongue in aspic	4b
1999-2000	France	10	3	3	Rillettes	4b
2000	Multiple states, USA	30	8	7	Delicatessen turkey ready-to-eat meats	1/2a
2000	North Carolina, USA	13	11	5	Home-made Mexican-style cheese	4b
2002	Quebec, Canada	17	3	0	Cheese made from raw milk	
2002	Multiple states, USA	54	12	8	Delicatessen turkey ready-to-eat meats	4b
2003	Texas, USA	12	7	3	Mexican-style cheese	4b

Source: Swaminathan and Smidt (2007).

2.6. Pathogenesis

Listeria species in particular *L. monocytogenes,* has the ability to sustain external pH, temperature, and salt stresses, which enables their survival under the acidic conditions within the mammalian stomach without being totally destroyed. Specifically, *L. monocytogenes* produces alternative sigma factor σB for regulation of several stress-response genes and related proteins (Dongyou, 2008).

Macrophages and epithelial cells are widely used to study the interaction of *L. monocytogenes* with mammalian host cells. However, it was shown that also neutrophils, dendritic cells, hepatocytes, fibroblasts, endothelial cells may become infected with, and serve as host cells for *L. monocytogenes in vitro* and *in vivo* (Bonazzi and Cossart, 2006). Then, *L. monocytogenes* synthesizes surface proteins called internalins (e.g., InA and InlB) to enable its entry into cells. Through its binding to an adhesion protein E-cadherin in host enterocytes leading to local cytoskeletal rearrangements, InlA facilitates *L. monocytogenes* entry into epithelial cells. By interacting with a hepatocyte growth factor receptor, InlB paves the way for *L. monocytogenes* entry into hepatocytes and other host cells. Entry occurs via zipper-like phagocytosis, characterized by the emission of small pseudopods that firmly entrap the bacteria and the intimate contact of the bacterial surface with the host cell plasma membrane. Inside the vacuoles, *L. monocytogenes* secretes listeriolysin (LLO) and phosphatidylinositol-phospholipase C (PlcA) to lyse the vacuolar membrane and escape to the cytosol, where it undergoes rapid intracellular expansion. Afterwards, *L. monocytogenes* generates another surface protein ActA and phosphatidylcholine-phospholipase C (PlcB) to aid its spread to neighboring cells. With the help of PlcB and a metalloprotease (Mpl), *L. monocytogenes* disrupts the secondary double-layer membrane vacuoles and initiates a new cycle of infection. The bacteria that are released into the cytoplasm begin to replicate while making use of specific transporters to gain carbohydrates from the host cell, whereas those remaining in the phagosome are killed and digested (Jemmi and Stephen, 2006). The virulence-associated proteins PlcA, LLO, Mpl, ActA, and PlcB are encoded by five adjacent genes, named *Listeria* Pathogenicity Island 1 or LIPI-1 (Dongyou, 2008).

2.7 Clinical Signs of listeriosis in humans

High numbers of *L. monocytogenes*, in excess of 100 colony forming units (CFUs), are required to cause invasive listeriosis upon oral exposure. Foods contaminated with less than100 CFUs/g, which corresponds to the numbers normally found in *Listeria*-contaminated retail food, are not considered to pose a risk to humans, even for individuals with increased susceptibility. Another factor that may obviously influence the MID is the virulence of the *L. monocytogenes* strain in question. Although all bacteria belonging to this species are assumed to be pathogenic, epidemiological evidence indicates that strain-to-strain differences in virulence exist. Thus, only 4 of the 13 *Listeria* serotypes—namely, 1/2a, 1/2b, 1/2c, and 4b—account for 98% of all cases of human and animal listeriosis worldwide(Dongyou, 2008). However, the most critical factor is the underlying condition and immunological status of the host as this determines the susceptibility to a given strain of *L. monocytogenes*. The vast majority of listeriosis patients have a physiological or pathological condition that impairs the capacity to mount an effective cellular immune response. Two major population groups at risk for invasive listeriosis are the neonates and the elderly (above 60 years), in which the immune system is immature or declining, respectively. Pregnant women, another major risk group, are assumed to be more susceptible to listeriosis due to the pregnancy associated depression of cell-mediated immunity. In non pregnant adults, almost all cases of listeriosis are seen in individuals with chronic, debilitating illnesses or subjected to immunosuppressive therapy. Specific risk groups in the intermediate-age band include cancer and organ transplant patients, HIV-infected and AIDS patients, and individuals with chronic liver disease (alcoholism and cirrhosis), diabetes, and lupus (Lunden *et al.*, 2004).

In summary, the severity and clinical outcome of *L. monocytogenes* infection depend on three principal variables: (1) the number of bacteria ingested with food; (2) the pathogenic properties and virulence of the infecting strain; and (3) the underlying condition and immunological status of the host. In immunocompetent individuals with no predisposing condition, ingestion of low to moderate doses of *L. monocytogenes* (less than 100 CFUs) has no effect other than boosting antilisterial protective immunity, whereas ingestion of large doses of the bacteria (greater than100 CFUs), sometimes doses as high as (1000) may cause acute febrile gastroenteritis within 24 h of consumption of the contaminated food due to massive invasion of the intestinal mucosa (Fedio and Jackson 1992)

2.7.1. Sepsis

Sepsis without a localized infection is the most common presentation in patients with deficient immune systems. The patient often appears severely ill with fever, nausea, vomiting and malaise (Doganay, 2003). Sepsis may progress to DIC, ARDS and multi-organ system failure. It requires a positive blood culture to establish a diagnosis (Dimaio, 2000).

2.7.2. Central nervous system infection

Central nervous system disease is the second most common presentation of *L. monocytogenes* infection in the immuno-compromised population, and the most likely presentation of listeriosis in the immunocompetant population. Meningitis is the commonest form of CNS listeriosis. Clinical features of listerial meningitis are similar to that of more common etiologic agents. The onset of infection may be acute or subacute. The clinical picture is usually characterized by high fever, nuchal rigidity, movement disorders such as tremor and/or ataxia, and seizures. Seizures are seen more commonly than in other types of meningitis (Doganay, 2003). The most common non-meningitic form of CNS listeriosis is encephalitis, involving the brainstem, of which infection is similar to circling disease found in sheep, named rhombencephalitis. Patients with listerial meningoencephalitis have subacute onset of illness that is characterized by focal neurological endings in the hindbrain, including ataxia and multiple cranial nerve abnormalities. Fever may be absent or unnoticeable in 15% of cases. In rhombo-encephalitis coma may be the initial symptom in up to 30% of patients and is seen most often in the older and more immunosuppressed population. The course of rhombo-encephalitis is usually so severe that patients either die or have serious permanent neurological disease. Recurrence is rare (Dimaio, 2000).

2.7.3. Endocarditis

Listerial endocarditis is responsible for 8–10% of all listerial infections. This manifestation is usually found in those with a prosthetic cardiac valve or those who have previously damaged and scarred valves. However, the organism has also been reported in native valve infections. *Listeria* has been found to preferentially infect left-side valves and is often a source of systemic bacterial emboli. The mortality rate for this infection is approximately 50 % (Dimaio, 2000).

2.7.4. Complications in pregnancy

Cell-mediated immunity is slightly decreased during pregnancy, and this alteration places the pregnant woman at risk for listerial disease. The infection is most commonly seen during the third trimester, which may be secondary to a further decrease in immune system function. However, the disease may occur at all stages of gestation. *Listeria monocytogenes* has a predilection for the placenta, which is often unreachable by the immune system. The disease usually presents with bacteremia, and the most common manifestations are fever (often greater than 39°C), headache, arthralgia, and malaise (Doganay, 2003). The bacteremia often results in hematogenous spread and translucent infection which in turn, may lead to chorioamnionitis, premature labor, premature rupture of membranes, or early-onset of infection in the neonate. Signs of intrauterine infection include diarrhea, nausea, vomiting, backaches, abdominal pain, and bloody vaginal discharge. The placenta is often found to have gross abscesses by visual inspection, as well as micro-abscess with necrosis by microscopy. The abscesses are usually multiple, well circumscribed, gray and solid (Dimaio, 2000; Farber and Peterkin, 1991).

If a mother becomes infected with *L. monocytogenes* the fetus is affected in most of the cases. Listeriosis results in stillbirth or neonatal death. Infection that occurs early in pregnancy is more likely to result in fetal death. Women, who are treated promptly with antibiotics during pregnancy, usually have decreased morbidity and mortality, a fact that highlights the importance of making an early and accurate diagnosis of listeriosis (G'rdhar and Garg 2002).

2.7.5. Neonatal infection

Listeria infection of the fetus may immediately result in spontaneous abortion or stillbirth. Listeriosis should be suspected when there is a spontaneous abortion and a fever preceded the fetal loss by 24–48 hours. The spectrum of disease can be divided into early- and late-onset listeriosis. Early-onset disease occurs within one week of delivery but usually manifests within two days postpartum. Early-onset disease is probably acquired in uterus and often presents in the preterm infant with sepsis, respiratory distress, purulent conjunctivitis and skin lesions. The highest inoculum of bacteria is usually found in the lung and gut, implicating infantiseptica, and it is characterized by widespread abscesses and granulomas on the skin and in the visceral organs of the neonate (Dimaio, 2000; Doganay, 2003). Late-onset of the disease is usually diagnosed 1–2 weeks postpartum and is most commonly found in full-term infants. The infants usually have uncomplicated deliveries, and the mode of transmission is probably during passage through the birth canal. The complications of perinatal infection are often grave (Doganay, 2003).

2.7.6. Gastrointestinal disease

In a healthy population, consumption of foods contaminated with *L. monocytogenes* may cause a self-limited syndrome presenting with fever, nausea, vomiting and diarrhea (Doganay, 2003).

2.7.7. Focal infections

Listeria not only causes systemic disease, but also localized infections such as conjunctivitis. These superficial infections are most commonly found in veterinarians and other animal workers. Listerial bacteremia has been implicated in a number of diseases and has been a cause of peritonitis, cholecystitis, hepatitis, pleuritis, splenic abscess, pericarditis, and osteomyelitis.

The above infections may be the result of septic emboli with listerial endocarditis. These infections are most commonly seen in immunocompromised patients (Doganay, 2003).

2.8. Diagnosis

Central nervous system symptoms, fever, sepsis, and food-borne or febrile gastroenteritis in neonatal, elderly, pregnant woman and immunocompromised individuals leads to the suspection of listeriosis. Diagnosis requires growth of the organism from body fluids that are normally considered sterile. The clinician should obtain blood, amniotic fluid and cerebrospinal fluid for culture if listeriosis is suspected. The bacteria will grow easily on routine culture medium within 24–36 hours and make biochemical testing important in identification of the species. Newly adapted tests with antibodies to LLO may assist in confirming the infection (Doganay, 2003)

2.8.1. Detection of *Listeria* species

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

Enrichment media

Media enhancing the growth of *Listeria* and suppressing the growth of other organisms are used. The selective enrichment broths in common use and/or recommended by regulatory bodies such as the FDA, the USDA and the International Organization for Standardization (ISO) are: *Listeria* enrichment broth (LEB), Fraser broth, and University of Vermont media which contains acriflavine and nalidixic acid (Aurora *et al.*, 2008).

Primary or pre-enrichment broth usually contains lower amounts of the selective agents to aid the resuscitation of possibly injured cells (Beume and Hazeleger, 2003). Acriflavine inhibits RNA synthesis and mitochondriogenesis and is commonly used in enrichment. Nalidixic acid inhibits the DNA synthesis of cells.

Isolation media

After selective enrichment, cultures are then plated on to selective/differential agar plates for isolation of presumptive colonies of *Listeria* species. Media used includes Oxford agar, modified Oxford (MOX) agar formulation, Lithiumchlorid/phenyl ethanol/ moxalactam (LPM) or PALCAM (Polymixine acriflavine lithiumchloride ceftazidine aesculin mannitol). Typical colonies of *Listeria* spp are small, black and surrounded by a black halo (OIE, 2004).

The development of blood-containing media allowed the separation of the hemolytic species from the non-hemolytic and non-pathogenic species. The most important features discriminating between *Listeria* spp commonly found in foods and food environments are hemolysis and sugar fermentation.

The introduction of chromogenic media efficiently improved the isolation of *L. monocytogenes*. In these media, differentiation of *L. monocytogenes* from other *Listeria* spp. is based on the production of a phosphotidylinositol-specific phospholipase C by *L. monocytogenes*, which hydrolyses a specific substrate added to the medium, producing an opaque halo around the colonies. It was observed that after prolonged incubation of 48hrs some *L. ivanovii* strains produced a weak halo as well. Substrates for the detection of β-glucosidase, common to all *Listeria* species, may be used as elective feature for the detection of all *Listeria* colonies. Lithium chloride, nalidixic acid and/or cycloheximide are added to the media to obtain sufficient selectivity (Beume and Hazeleger, 2003).

Confirmation and identification

Typical colonies, which are confirmed by microscopy (Gram-positive bacilli or coccobacilli) and tests for catalase- (+) and oxidase-activity (−), have to be tested for hemolytic activity to distinguish between *L. monocytogenes* and *L. innocua*, the two species most frequently isolated. For further identification, to species level biochemical tests are used, including hemolysis and fermentation of a range of sugars. Preference should be given to those tests that differentiate *L. monocytogenes* from non-hemolytic species (Gasanov *et al.*, 2005).

Rapid methods

Since traditional methods for the recovery and identification of pathogens in food are both time consuming and labour intensive, rapid and/or automated detection methods have been developed. Food producers and distributors as well as public health authorities have great interest in rapid methods. In principle, these methods permit a more efficient control of raw materials, processes and products and may play an important role in food trade and product liability. Therefore, it is important that test results obtained with newly developed rapid methods are reliable and will be accepted by all parties involved. General acceptance of a new method will only be realized if extensive testing and validation have shown the method to be sufficiently sensitive, specific, user friendly and not too expensive. Recently, new methods are described at a high rate particularly methods based on in vitro RNA amplification (an accurate indicator of cell viability) with the use of the polymerase chain reaction (PCR) are available (Aurora *et al.*, 2008).

2.9. Treatment and Control

2.9. 1. Treatment

Several antibiotics including Ampicillin, gentamicin, Tetracycline, Penicillin, Amoxicillin, trimethoprim-sulfamethoxazole, Tetracycline, Kanamycine and Vancomycine are of the treatment of choice for listeriosis. All patients should be treated with doses high enough to penetrate the CNS, regardless of whether the patient has obvious signs of CNS involvement, because of the high affinity of *L monocytogenes* for these tissues. Treatment should be started immediately (Dimaio, 2000).

2.9.2. Control

Control and prevention strategies:

Listeria species, especially *L. monocytogenes*, are tolerant of extreme temperature, pH, and osmotic conditions, which are behind the principal mechanisms of food processing practices. Generally food products provide a nutrient-rich medium for *Listeria* maintenance and multiplication, and any viable *Listeria* bacteria that have survived the food processing procedures will likely grow and cause infection if not completely eliminated or reduced prior to consumption. WHO had formulated three lines of defense against *Listeria* (WHO, 1988).

Before Processing

The first approach focuses on controlling *Listeria* in the food-producing animal (pre-harvest control). Pre-harvest control at the farm level has long been considered an important part of pathogen reduction schemes. Therefore, it is fundamental that monitoring programmes should be established to identify infected herds and animals and that efforts are made to find and control the sources of infection and prevent further spread. The ultimate objective is to produce disease free animals. According to several studies, improving hygiene and husbandry management programmes, including feed control are of major importance in aiding animals to withstand exposure to bacteria, and to minimize the possible subsequent spread of the agent on the farm (Farber *et al*; 1989).

During Processing

The second approach involves improving hygiene during processing of products (harvest control). Appropriate milking and hygiene procedures need to be developed and strictly followed to achieve any reduction in *Listeria* contamination (WHO, 1988). Direct contact with contaminated processing equipment is an important cause of *L. monocytogenes* occurrence in food products. Adoption of HACCP by food processing companies during processing (e.g., thorough cleansing of in-process products and food-contact surfaces, clear separation of staff functions, and good personal hygiene) has led to significant reduction of *L. monocytogenes* contamination in finished food products. In particular, use of heat treatment (e.g., hot steam, hot air, or hot water at 80°C) is effective in cleaning and sanitizing equipments and in eradicating *L. monocytogenes* from food processing plants. In addition, visitors and staff job rotation are potential risk factors for increasing *L. monocytogenes* contamination in food processing plants. Optimization and application of more effective and innovative combinations of detergents and sanitizers may also help eliminate *L. monocytogenes* in food processing environments. For example, Cloat *et al* (1989) reported that an alkaline treatment (pH 10.5) followed by an acid treatment (pH 5.4) provided a more efficient means of killing *L. monocytogenes* than other combinations of sanitizers and detergents.

After Processing

The third approach targets the final preparation of food by educating the food industry, suppliers, retailers and consumers about good hygiene practices (post-harvest control). During processing, raw food of animal origin undergoes different treatments intended to inhibit the multiplication of microorganisms or even to eliminate them fully. Factors such as the cross contamination of final products by raw materials, failure during heat treatments or other decontamination processing, inadequate refrigeration and poor personal hygiene of food handlers are well documented and will contribute to outbreaks of food borne listeriosis (WHO, 1988).

Implementation of monitoring and quality control procedures represents the most cost-effective post processing measures against food-borne pathogens, including *L. monocytogenes*. Besides assisting the formulation of cleaning and sanitizing programs to reduce and eradicate *L. monocytogenes* in food processing environments, these procedures help ensure that the finished food products meet the recommended tolerable levels of *L. monocytogenes* in the specified product categories (100 colony forming units per gram foodstuff) before releasing to the retail markets. Finally, there is scope for continued improvement in home food-handling practices, where storage of foods at incorrect refrigeration temperatures often contributes to heightened risk of listeriosis in humans. Generally the successful prevention of foodborne listeriosis originating from animal production must involve all the three lines of defense.

3. MATERIALS AND METHODS

3. 1. Study area

The study was carried out in Debre Zeit town between October 2008 and April 2009. Debre Zeit is located at 9 ^{0} N and 40 ^{0} E. It is 47 km south east of Addis Ababa, with human population of about 95,000. Its altitude is about 1850 m above sea level. It experiences bimodal patterns of rainfall with the main rainy season extending from June to September. A short rainy season occurs between March and May with an average rainfall of about 800 mm. The mean annual minimum and maximum temperatures are 12.3 ^{0}C and 27.7 ^{0}C, respectively with an overall average of 18.7 ^{0}C (CSA, 2003). Highest temperatures are recorded in May. The mean relative humidity in Debre Zeit is 61.3%.

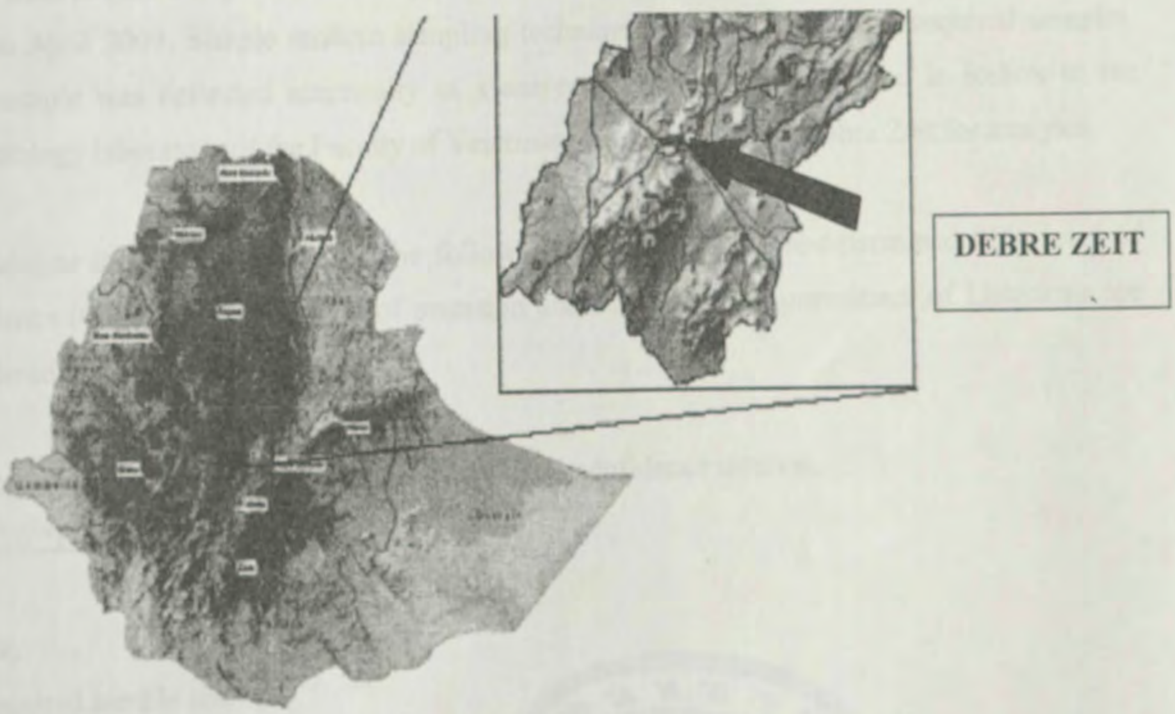

Figure 1: Map of study area

3.2. Origin and type of samples

Ada Co-operative has fifteen collection centers collecting raw milk from one hundred farms. The collection center was selling raw and pasteurized milk to individuals and super markets. In the current study, eight collection centers were selected randomly to collect bulk raw milk samples and twenty five farms were selected randomly to collect farm raw milk samples. An open local market in Debre Zeit town was visited weekly to collect cottage cheese samples. Cottage cheese or "Ayib" is an Ethiopian traditional dairy product made from sour milk after the removal of the fat by churning and cooking the curd to a temperature of 40^0C to 70^0C.

3.3. Study Design and Sample Size

A cross-sectional study was conducted in Ada Dairy collection center, different Dairy farms that supply milk to Ada Dairy collection center and an open local market in Debre Zeit from October 2008 to April 2009. Simple random sampling technique was used to collect required samples. Each sample was collected aseptically in a universal bottle and transported in icebox to the microbiology laboratory of the Faculty of Veterinary Medicine, AAU, Debre Zeit for analysis.

To calculate the total sample size, the following parameters are pre-determined 95% level of confidence (CL), 5% desired level of precision and 50% expected prevalence of Listeriosis are considered to find the sample size.

The formula used was (Thrusfield, 2005) with 95% confidence interval.

$$n = \frac{1.96^2 * P_{exp} * (1-P_{exp})}{d^2}$$

Where,

n = required sample size

Pexp = expected prevalence

d = desired absolute precision.

Using the above formula, the maximum sample size is 384. But a total of 400 samples were collected in this study as shown in table5. The samples were processed immediately or stored over night in a refrigerator at -20°C.

Table 5: Type and number of samples collected during the study

Serial number	Types of samples collected	Number of samples examined
1	Farm raw milk	100
2	Bulk raw milk	100
3	Cottage cheese	200

3.4. Isolation and identification of *Listeria*

For the isolation and identification of *Listeria* species in milk and cheese samples, the techniques recommended by the International Organization for standardization (ISO 11290-1: 1998) were employed.

3.4.1. Primary selective enrichment

The primary selective enrichment step involves a selective liquid media with reduced concentrations of selective agents was a half Fraser broth. Twenty-five Grams (25 ml) of each sample was added to a stomacher bag containing 225 ml of half Fraser broth to obtain a ratio of 1:9. The mixture was homogenized using a laboratory blender (Stomacher 400, Seward, England) at high speed for 2 minutes. The mixture was incubated at 37°C for 24 hours.

3.4.2. Secondary selective enrichment

The secondary selective enrichment medium (Fraser broth: AES Lab., COM Bourg, France) with full concentration of selective agents was employed. From the pre-enrichment culture (half Fraser broth), 0.1 ml was transferred into 10 ml of Fraser broth The medium was incubated at 37°C for 48 hours (Vitas and Garcia, 2004).

3.4.3. Plating and identification

From the secondary enrichment culture, Fraser broth a loop full of the culture was taken and streaked onto sterile PALCAM (polymixin acriflavine lithium chloride ceffazidine aesculine mannitol) agar plates and Oxford agar plates (AES Lab., COM Bourg, France) and incubated at 37°C for 24 to 48 hours based on the growth of colonies. The plates were examined for the presence of characteristic colonies presumed to be *Listeria*. On PALCAM agar, of being small or very small grayish green or Olive green colonies of 1.5-2mm in diameter, and some times with black centers but always with black halos. After 48 hours colonies become darker, with a possible greenish sheen and are about 2mm in diameter with black halos and sunken centers. On Oxford agar, *Listeria* hydrolyses esculin to esculetin and forms a black complex with iron ions. Therefore, *Listeria* form brown-green coloured colonies with a black halo (Quinn *et al* 1999).

3.4.4. Confirmation

Five Colonies suspected to be *Listeria* were transferred onto pre-dried plates of trytic Soya yeast extract agar (TSYEA) (Difco, Bacton, USA), which allow well-separated colonies to develop. All colonies were taken for confirmation when fewer than five. The plate is then incubated at 37°C for 24 hours or until growth is satisfactory. Typical colonies were grown on trytone Soya yeast extract agar (TSYEA). The colonies are 1mm-2mm in diameter, convex, colour less and opaque. For further confirmation biochemical tests were done.

Catalase reaction

Isolated colonies obtained in TSYEA were transferred onto clean and sterile glass slide and a drop of 3% hydrogen peroxide (Sigma, steinheim, Germany) solution was added on the glass slide. The immediate formation of gas bubbles was an indication of a positive reaction.

Gram staining

Gram staining was performed on separate *Listeria* colonies taken from TSYEA.

Motility

Colonies from TSYEA were taken and inoculated into SIM (hydrogen sulphide, indole and motility) medium (Becton Dickinson, Maryland, USA) using strait inoculating loop and incubated for 48 hours at 25°C. A typical umbrella like growth pattern under the sub surface was an indication of motility (Quinn *et al* 1999).

Hemolysis test

A sheep blood agar plate was inoculated with isolated colonies taken from TSYEA using an inoculating loop to determine the hemolytic reaction. The colonies were examined after incubation at 37°C for 24 hours for the presence of hemolysis.

Carbohydrate utilization tests

After inoculation of Tryptone soya yeast extract broth (TSYEB) with *Listeria* colonies it was incubated at 37°C for 24 hours. The carbohydrate utilization broth (rhaminose, xylose, and mannitol) were prepared using phenol red as an indicator and inoculated with a culture from TSYEB and incubated at 37°C for up to 5 days (ISO 11290-1, 1998). Positive reactions (acid formation) were indicated by a yellow colour that developed within 24 to 48 hours (Quinn *et al* 1999).

CAMP (Christine Atkins Muench Peterson) test

Each of a known ß-hemolytic *Staphylococcus aureus* was streaked in a single line across sheep blood agar plate. The test strain or the culture was streaked in a similar fashion at right angle so that the test cultures and *Staphylococcus aeureus* do not touch but closer about 1mm-2mm apart.

The plates then were incubated at 37°C for 18-24 hours (Quinn *et al* 1999). An enhanced zone of ß- hemolysis at the intersection of the test strains with of *S. aeurus* was considered to be a positive reaction (Gasanov *et al.*, 2005). The summarized procedure for isolation and identification of *Listeria* is indicated.

Isolation and identification of *Listeria*

⇩

Pre enrichment stage

Twenty-five Grams (25 ml) sample will be added in a stomacher bag containing 225 ml of half Fraser broth

⇩

The mixture will be homogenized using a laboratory blender

⇩

The test portion will be incubated at 37°C for 24 hours⇩

Enrichment stage

0.1 ml will be transferred into 10 ml of Fraser broth

(Secondary enrichment)

⇩

The medium will be incubated at 37°C for 48 hours

⇩

Plating stage

A loop full of the culture will be taken and streaked on to sterile PALCAM and Oxford agar plates

⇩

Then it will be incubated at 37°C for 24 to 48 hours

⇩

Five Colonies suspected to be *Listeria* would be transferred on to TSYEA

⇩

Then incubated at 37°C for 24 hours

⇩

For confirmation

Catalase Test

3% hydrogen peroxide

⇩

Gram staining

⇩

Motility

SIM medium

⇩

Hemolysis test

Sheep blood agar

⇩

Carbohydrate utilization tests

Rhaminose, xylose, and mannitol

⇩

CAMP (Christine Atkins Muench Peterson) test

A known ß-hemolytic S. aureus and R. equi

3.5. Data management and analysis

The data were entered and managed in MS Excel and analysis was conducted using SPSS Software versions. Prevalence estimation of *Listeria* in different sample types was expressed as percentage, with 95% confidence interval (CI) and prevalence was determined by number of positive food samples divided by the total number of food samples examined. Difference between positive proportions was determined by Pearson's Chi-square ($\chi 2$) test. P of less than 0.05 was used to see the level of significance.

4. RESULTS

After sequential procedures for the isolation and identification, organisms with typical morphological and biochemical characteristics were considered positive for *Listeria* species as indicated on table 6.

Table 6: Morphology and biochemical characteristics of samples positive for *Listeria* species

Medias or Biochemical tests	*L. monocytogenes*	*L. ivanovii*	*L. seeligeri*	*L. innocua*	*L. welshimeri*	*L. grayi*
Oxford agar	Brown-green coloured colonies with black centers& black haloes					
PALCAM agar	Small grayish green or Olive green with black center& black haloes					
Catalase	Bubble formation					
Hemolysis	Weak zone of hemolysis	Wider zone of hemolysis	Weak zone of hemolysis	No hemolysis	No hemolysis	No hemolysis
CAMP test on *S .aeurus*	Enhanced zone of hemolysis	No enhanced zone of hemolysis	Weak enhanced zone of hemolysis	CAMP test negative	CAMP test negative	CAMP test negative
Rhaminose fermentation	+ (Yellow colour)	-(Red colour)	+ (Yellow colour)	±(Red or Yellow)	±(Red or Yellow)	±(Red or Yellow)
Xylose fermentation	-(Red colour)	+ (Yellow colour)	+ (Yellow colour)	-(Red colour)	+ (Yellow colour)	-(Red colour)
Manitole fermentation	-(Red colour)	-(Red colour)	-(Red colour)	-(Red colour)	-(Red colour)	+ (Yellow colour)

Out of a total of 400 raw milk and cheese samples examined, 49 (12.25%) samples were positive for *Listeria* species. One hundred farm raw milk samples collected were 16% positive and one hundred bulk raw milk samples were 27% positive. Two hundred cottage cheese samples hundred bulk raw milk samples were 27% positive. Two hundred cottage cheese samples collected from an open market were 6 (3 %) positive for *Listeria*. There was no statistical significance between farm raw milk and bulk raw milk because P= 0.058. The overall prevalence of *Listeria* species in different sample is summarized in Table 7.

Table 7: Distribution of *Listeria* isolated in raw milk and cottage cheese samples

Number and percentage of *Listeria* isolated from different sample types				
Type of sample examined	Farm Raw milk	Bulk Raw milk	Cottage Cheese	Total
Total positive	16	27	6	49
Samples examined	100	100	200	400
Prevalence (%)	16	27	3	12.25
95% confidence interval	8.7-23.3	18.4-35.6	0.6-5.4	9.2-15.4
P value	P=0.058			

The prevalence of *Listeria* spp in cottage cheese (3%) was lower than for raw milk samples (16%-27%). Bulk raw milk prevalence (27%) was higher than farm raw milk prevalence (16%).

4.1. *Listeria* species observed from farm raw milk samples

From a total of 100 farms raw milk samples *Listeria monocytogenes* had the highest prevalence (5%) followed by *L. innocua* (4%) and *L. ivanovii* (3%). *Listeria welshimeri* had 2% prevalence while *L. seeligeri and L. grayi* had 1% prevalence each (figure 2).

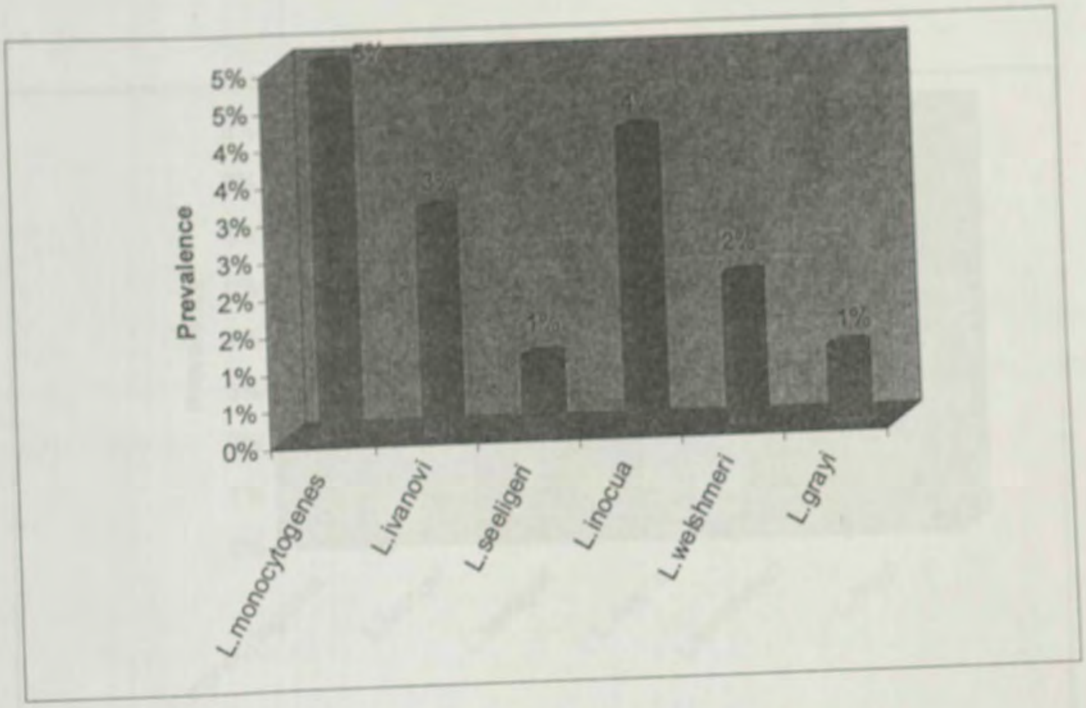

Figure 2: The distribution of *Listeria* species in farm raw milk samples

4.2. *Listeria* species observed from bulk raw milk samples

From a total of 100 bulk raw milk samples, *L. inocua* was highly prevalent (8%) followed by *L. monocytogenes* (7%) and *L. ivanovii* (4%). *Listeria seeligeri and L. grayi* had 3% prevalence each and *L. welshimeri* 2%. In the over all 27 (27%) were positive. The distributions of different *Listeria* species in bulk raw milk are depicted in Figure 3.

4.3. *Listeria* species observed from cottage cheese samples

Six samples (3%) were positive for *Listeria* out of 200 cottage cheese samples. *Listeria innocua* had 1.5% prevalence followed by *L. monocytogenes* 1% and *L. ivanovii* 0.5%. The distributions of these *Listeria* species in cottage cheese are shown in Figure 4.

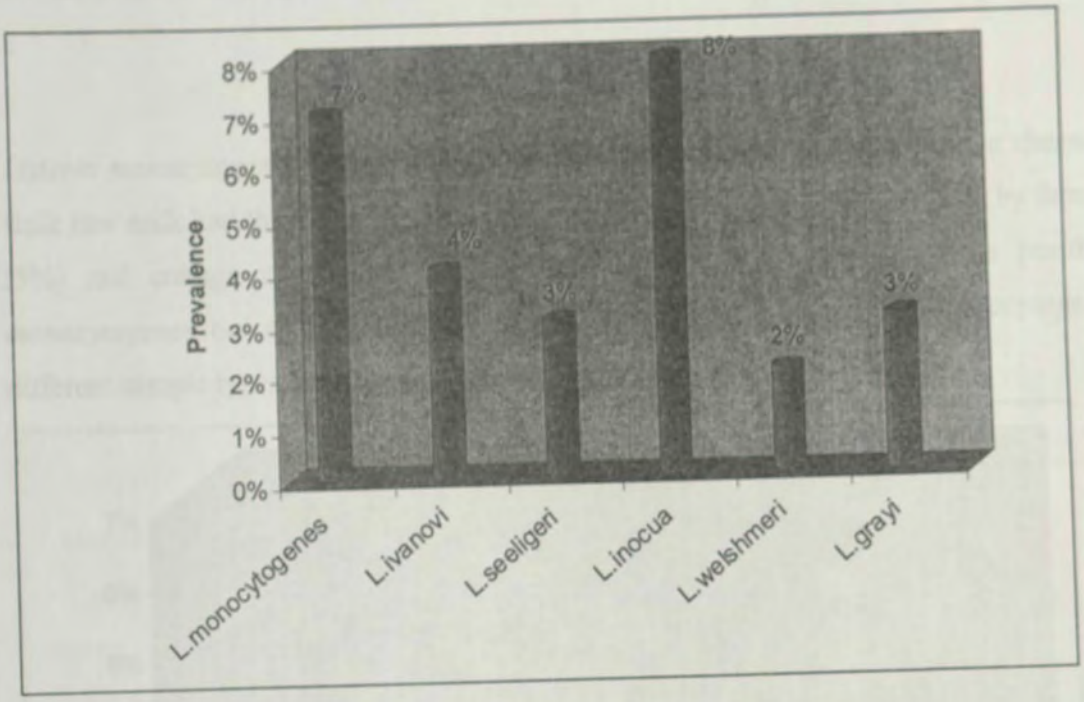

Figure 3: The distributions of *Listeria* species bulk raw milk samples

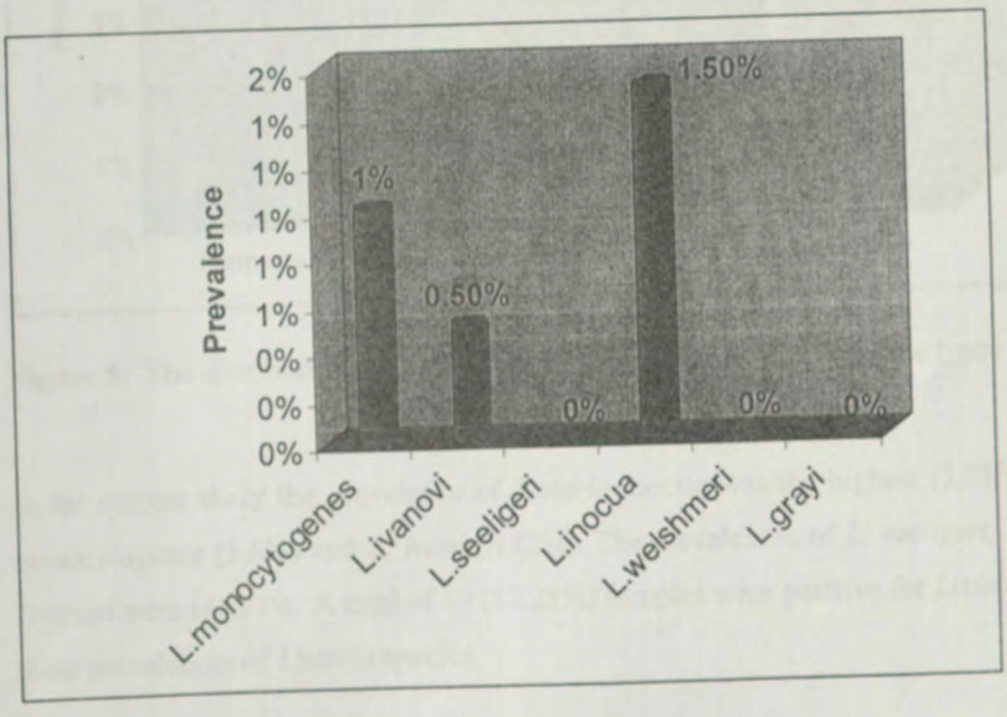

Figure 4: The distributions of *Listeria* species in cottage cheese samples

4.4. *Listeria monocytogenes* observed from raw milk and cottage cheese samples

Listeria monocytogenes was more prevalent in raw milk samples than cottage cheese samples. Bulk raw milk had the highest prevalence of *L. monocytogenes* (7%) followed by farm raw milk (5%) and cottage cheese (0.5%). A total of 13 (3.25%) samples were positive for *L. monocytogenes* out of the total 400 samples. The distributions of *L. monocytogenes* in the different sample types are shown in Figure 5.

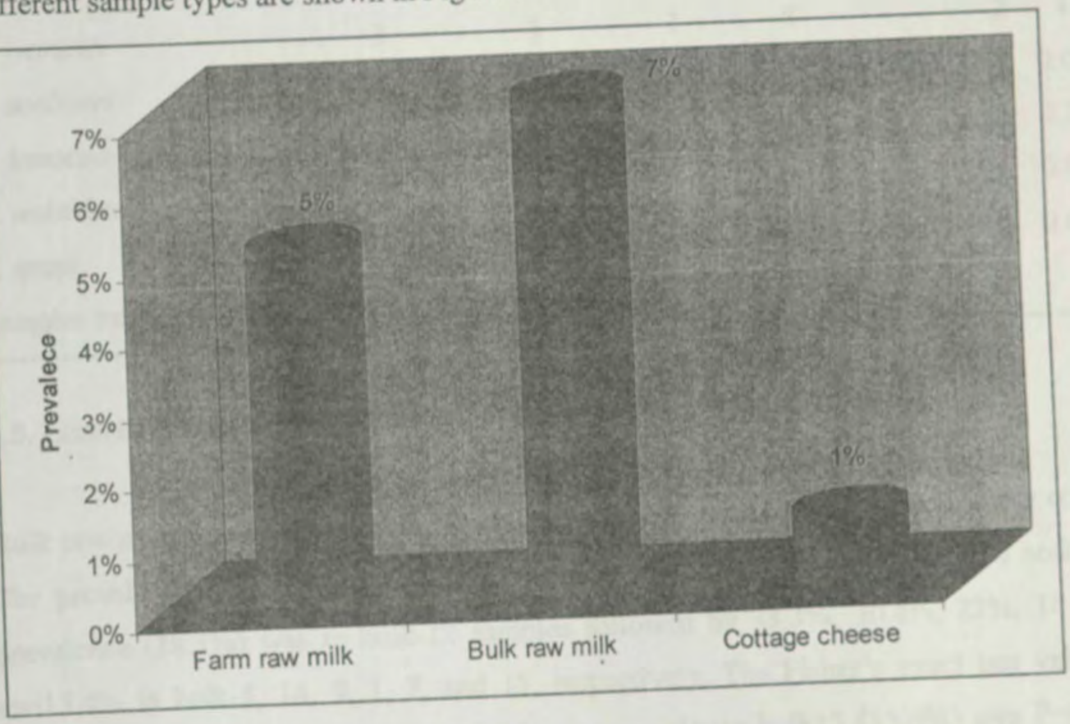

Figure 5: The distribution of *Listeria monocytogenes* in different sample types

In the current study the prevalence of *Listeria inocua* was the highest (3.75%) followed by *L. monocytogenes* (3.5%) and *L. ivanovii* (2%). The prevalences of *L. seeligeri, L. welshimeri and L. grayi* were each 1%. A total of 49 (12.25%) samples were positive for *Listeria*. Table 8 depicts these prevalences of *Listeria* species.

Table 8: Distribution of different *Listeria* species isolated in raw milk and cottage cheese samples

Listeria species isolated	Number and percentage of *Listeria* species isolated from different sample types					
	Farm Raw milk	Bulk Raw milk	Cottage Cheese	Total	Prevalence (%)	95% confidence interval
L. monocytogenes	5	7	2	14	3.5	1.7-5.3
L. ivanovii	3	4	1	8	2	1.3- 2.7
L. seeligeri	1	3	-	4	1	0.025-1.99
L. innocua	4	8	3	15	3.75	1.85- 5.65
L. welshimeri	2	2	-	4	1	0.025-1.99
L. grayi	1	3	-	4	1	0.025-1.99
Samples examined	100	100	200	400		

4.5. *Listeria* from different collection centers in Ada Dairy Co-operative

Bulk raw milk was collected in eight of the fifteen collection centers in Ada Dairy co-operative. The prevalence of *Listeria* was highest (41.6%) in bulk-2 milk samples. The second highest prevalence (38.5%) was in bulk-12 samples followed by 33.3%, 30.8%, 23%, 16.7%, 16.7% and15.4% in bulk-4, 14, 9, 1, 7, and 15, respectively. The Fisher's exact test value between highest prevalence bulk 2 (41.6%) and lowest prevalence bulk15 (15.4%) was P=0. 202. The prevalences of the *Listeria* species obtained in eight bulk raw milk samples are indicated in Table 9.

Table 9: Prevalence of *Listeria* species in eight different bulk raw milk samples

Bulks examined	Bulk 1	Bulk 2	Bulk 4	Bulk7	Bulk 9	Bulk 12	Bulk 14	Bulk 15
Total positive	2	5	4	2	3	5	4	2
Samples examined	12	12	12	12	13	13	13	13
Prevalence (%)	16.7	41.6	33.3	16.7	23	38.5	30.8	15.4
95% confidence interval	0.04-0.38	0.14-0.7	0.06-0.6	0.04-0.38	0.11-0.35	0.12-0.66	0.06-0.56	0.04-0.34
P value	P (value between bulk 2 and 15)=0.202							

5. DISCUSSION

Food-borne listeriosis was suggested early in medical literature but was first demonstrated in 1981 during an outbreak in Canada (Axelsson and Sorin, 1998). Since then epidemiological investigations have repeatedly indicated that the consumption of contaminated food is the primary vehicle of transmission of listeriosis and different foods have been identified as the vehicles of several major outbreaks of listeriosis. It is an emerging disease with a low incidence but, with a high fatality rate especially in immuno-compromised individuals (Norrung, 2000). The severity and case-fatality rate of the disease require appropriate preventive measures but, the characteristics of the microorganism are such that it is unrealistic to expect all food to be *Listeria* free. This dilemma has created a momentum for research in various areas such as conventional and rapid methods of detecting *L. monocytogenes* in foods (Rocourt and Cossart, 1997).

Listeria organisms are present in many food products both raw and processed. They are able to survive in these products in storage conditions. A survey by the Public Health Laboratory Service, London, England (McLauchlin and Gilbert, 1990) indicated that 6% of 18,000 food samples examined had *L. monocytogenes*. In another study, 11% of foods sampled from the refrigerators of patients suffering from listeriosis in the United States were positive for *L. monocytogenes* (Pinner *et al*., 1992). In addition to the above reports, Rorvik and Yndestad (1991) found in total of 460 different retail food items in Norway, 78 (16.9%) were contaminated with *L. monocytogenes.* Choi *et al.* (2001) also found that from a total of 410 domestic Korean food samples analyzed for the presence of *Listeria* species 8 (1.95%) were contaminated with *L. monocytogenes.*

In Ethiopia, Molla *et al*. (2004) reported an overall prevalence of 32.6% for *Listeria* species and 5.1% were *L. monocytogenes* from a total of 316 different retail food samples examined in Addis Ababa. Mengesha (2005) reported from a total of 711 different food samples examined, 189 samples were positive for *Listeria* species with an overall prevalence of 26.6% and *L. monocytogenes* was the second predominant species with an overall prevalence of 4.8% in Addis Ababa.

In the current study an over all prevalence of 12.5% out of 400 samples was obtained for *Listeria* and *L.monocytogenes* had a prevalence of 3.5%. The prevalence of *L. monocytogenes* in the current study lies with in the ranges of different findings recorded in various countries and this suggests that there is a potential risk of contracting *L. monocytogenes* through consumption of these milk and milk products in Ethiopia. Entry of *L. monocytogenes* in the food chain might occur through animals which excrete the bacterium, contaminated workers' shoes and clothing, contaminated surfaces or equipment and possibly through healthy human carriers (Rocourt and Cossart, 1997).

In this study, the organism isolated might have come from cows that were milked or it might have come from the environment including working personnel in milking area. Contaminated equipments or personnel in the market could also be the possible sources of contamination of cottage cheese in the market.

Recent outbreaks of listeriosis in humans have confirmed the potential for milk and milk products of being vehicles of *L. monocytogenes* (Lanciotti *et al.*, 1999). Milk can be contaminated by environmental sources including cow dung, soil, straw and more rarely, by mastitis. Cows suffering from mastitis caused by *L. monocytogenes* are very rare, nevertheless, raw milk must be considered as it's a source from contamination (Rocourt and Bille, 1997). World Health Organization (WHO, 1988) also indicated that the origin of *L. monocytogenes* in milk is mainly from fecal contamination. Fedio and Jackson (1992) also found in a comparative study of 3 farms with no history of *L. monocytogenes* the presence of this organism in 13.2% rectal fecal samples although there was no report of its finding in raw bulk milk tank from these farms.

Study done in Hungary by Reka *et al.* (2004) obtained 26.1% prevalence of *Listeria* in raw milk. Another study done in Spain by Dominguez *et al.* (1995) indicated ever a higher prevalence of 45% in raw milk. Farber *et al.* (1998) in Canada and Moura *et al.* (1993) in Brazil both indicated prevalences of 12% and 13% respectively in raw milk. The current study done on Ada bulk raw milk and farm raw milk revealed a prevalence of 24% and 16% for *Listeria*. *Listeria monocytogenes* was isolated in 7% and 5% of study samples respectively. The results of the current study of 24% and 16% prevalence are in line with results of 12% and 45% by Farber *et al*. (1998) in Canada and Dominguez *et al*. (1995) in Spain.

The difference in the prevalence of *L. monocytogenes* in foods in different countries might be attributed to the difference in food item composition or the difference in the level of hygiene and sanitation in food chain. Furthermore, the probable difference in the sensitivity of bacteriological detection methods could also be a major cause of difference

Acidified dairy products such as cottage cheeses are, in principle said to be free of *L. monocytogenes* (WHO, 1988). This might be due to the way cheese is prepared and thus the reason for lowest prevalence of this organism in this product. But due to the ability of *Listeria* to survive a higher pH and wider temperature ranges it is possible to find the bacteria in cottage cheese. *Listeria* is ubiquitous in nature and is found every where in the environment and hence contamination of cottage cheese could occur from cow dung, soil, personnel preparing the cheese and equipments utilized. Study done in Hungary by Reka *et al* (2004) indicated a1.8% prevalence. Molla *et al.* (2004) in Ethiopia indicated 1.8% of cottage cheese was contaminated with *Listeria* species. The result of the current study of 3% is a little bit higher than the finding of these researchers which may be due to poor hygienic conditions during preparation, after preparation and also in the market.

In this study the prevalence of *Listeria* species was highest for *L. innocua* (3.75%) which is said to be frequently isolated *Listeria* species. The second one was for *L. monocytogenes* (3.5%) which causes zoonotic listeriosis. The risk of this zoonotic pathogen is significant where the number of immunocompromised people (new born, elderly, pregnant women and HIV patients) is very high. This is because these people are considered of risk groups for infection with *L. monocytogenes* (WHO, 1988).

The result of eight of the bulk milk examined indicated bulk 2 had the highest prevalence (41.6%) and bulk 15 had the lowest prevalence (15.4%). Fisher's exact test showed p= 0.202 which was greater than 0.05 and had no statistical significance. The variation was only due to chance. In the study of bulk raw milk, the prevalence of *Listeria* throughout study period was almost similar (25%) except the 4th and 8th week (37.5%). This gives a clue that the organism is ubiquitous in nature and found every where at any time.

A number of outbreaks and sporadic cases of listeriosis have been recorded through consumption of different kinds of food items contaminated with *L. monocytogenes* in other countries where the presence of this pathogen in foods was frequently reported. There are no published reports of outbreaks or sporadic cases of listeriosis, which can be attributed to *L. monocytogenes* in Ethiopia. However, this should not be interpreted as if foodborne listeriosis is not a risk in the country where there are millions of high risk groups including HIV/AIDS patients.

6. CONCLUSIONS AND RECOMMENDATIONS

Based on the study on prevalence of *Listeria* species on raw milk and cottage cheese in Debre Zeit we can conclude that:

Listeria monocytogenes was isolated in raw milk and cottage cheese in Debre Zeit.

The prevalence of *L. monocytogenes* in these products can be considered as a risk for consumers of food-born listeriosis since the foods are consumed raw under Ethiopian condition.

Prevalence of *Listeria* species in raw milk originated from different area was almost similar.

This study has also indicated that the farmers and Dairy processing plants are following poor hygienic procedures, which enabled *Listeria* species to contaminate raw milk and cottage cheese.

Based on the above conclusions the following recommendations are suggested.

Responsible veterinary authorities should educate farmers about hygienic handling of milk and milk products.

Monitoring hygienic programmes should be established to identify critical control points and efforts made to control the sources of infection with ultimate objective of reducing contamination of animal edible products like milk and cheese.

Responsible veterinary and public health authorities should educate food producer's suppliers and retailers about methods of production of microbiologically safe food and about the sanitary and hygienic methods of food storage and handling.

The food processing plants should be made aware of the principles of HACCP so that they produce safe food products.

Food producers, suppliers and retailers should also be monitored and inspected regularly by the responsible institutions so that they could supply safe food to the consumer.

It will also be very important to raise public awareness through various ways about the methods of safe food handling.

Further investigation should be conducted in different food items commonly consumed by the population at national level for *Listeria* species specially *L. monocytogenes*.

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8. ANNEX

Annex 1: Sample collection sheet for bacteriological analysis

Serial number	Source	Type and number of samples examined			Total	remark
		Farm raw milk	Bulk raw milk	Cottage cheese		
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
14						
15						
16						

Annex 2: Composition and preparation of culture Media used for the Laboratory analysis of the study sample.

A) Primary selective enrichment media (half Fraser) (AES Lab., Combourg, France)

Composition	g/l
Meat peptone (peptic digest of animal tissue)	5.0
Beef extract	5.0
Yeast extract	5.0
Sodium chloride	20
Disodium hydrogen phosphate dehydrate	9.6
Potassium hydrogen phosphate	1.35
Aesculine	10
Lithium chloride	3.0
Acriflavine hydrochloride	0.0125
Nalidixic acid	0.01

Directions:

Add 55.0 g of powder into one litter of distilled water

Mix carefully until complete dissolution.

Sterilize at 121°C for 15 minutes (N.B do not over heat).

Aseptically add 0.5 g of ferric ammonium citrate after filter sterilization.

B) Secondary selective enrichment medium (Fraser broth) (AES Lab., Combourg, France).

Composition	g/l
Meat peptone	5.0
Tryptone (Peptic digest of casein)	5.0
Beef extract	5.0

Yeast extract	5.0
Sodium chloride	20
Disodium hydrogen phosphate dehydrate	9.6
Potassium hydrogen phosphate	1.35
Aesculine	10
Lithium chloride	3.0
Acriflavine hydrochloride	0.02
Nalidixic acid	0.02

Directions:

Add 55.0 g of powder into one litter of distilled water

Mix carefully until complete dissolution.

Sterilize at 121°C for 15 minutes (N.B do not over heat).

Aseptically add 0.1 g of ferric ammonium citrate after filter sterilization.

Dispense in to sterile test tube.

C) Polymixin acriflavin Lithium chloride ceftazidime aesculine mannitol (PALCAM): *Listeria* selective plating out medium agar base (AES Lab., Combourg, France).

i) Agar base (AES Lab., Combourg, France)

Composition	g/l
Peptones	23
Starch	1.0
Sodium chloride	5.0
Yeast extract	3.0
Glucose	0.5
Mannitol	10
Aesculine	0.8
Ferric ammonium citrate	0.5
Phenol red	0.008

Lithium chloride	15.0
Agar	15.0

ii).Supplement for 1000ml medium (one vial) (AES Lab., Combourg, France)

<u>Composition</u>	<u>g/l</u>
Polymixine B sulphate (100,000u)	0.1
Acriflavine hydrochloride	0.05
Sodium cefazidime pentahydrate	0.116

Reconstitute the vial of supplement with 5ml of sterile distilled water.

iii). <u>Directions for complete medium</u>

Add 73.9g of powder in to one litter of distilled water.

Mix carefully and heat with frequent agitation and boil till the powder dissolves completely.

Sterilize at 121°C for 15 minutes (N.B do not over heat).

Cool it to about 45°C-50°Cand add 2impulse of the constituted PALCAM supplement.

Mix carefully and dispense in to sterile petridishes.

D) Oxford *Listeria* selective plating-out media

ii) Agar base (AES Lab., Combourg, France)

<u>Composition</u>	<u>g/l</u>
Proteose peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar (depending on the gel strength of the agar)	8-9
Aesculin	1.0
Ammonium iron	0.5
Lithium chloride	15.0

ii). Supplement for 1000ml medium (one vial) (AES Lab., Combourg, France).

Composition	g/l
Cyclohexamidine	400.0
Colistin sulphate	20.0
Acriflavine hydrochloride	5.0
Cefotetan	2.0
Fosomycin	10.0

Reconstitute the vial of supplement with 5ml of sterile distilled water.

iii). Directions for complete medium

Add 57.5g of powder in to one litter of distilled water.

Mix carefully and heat with frequent agitation and boil till the powder dissolves completely.

Sterilize at 121°C for 15 minutes (N.B do not over heat).

Cool it to about 45°C-50°Cand add 2impulse of the constituted PALCAM supplement.

Mix carefully and dispense in to sterile petridishes.

E) Tryptone Soya-yeast extract agar (TSYEA) (Difco, Detroit, USA).

Composition	g/l
Tryptone	17.0
Soya peptone	3.0
Sodium	5.0
Dipotassium phosphate	2.5
Glucose	2.5
Agar	15
Yeast	6.0

Directions:

Suspend 40g of the powder in to one litter of distilled water.

Mix thoroughly.

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

Autoclave at 121°C for 15 minutes.

Dispense in to sterile petridishes.

F) Tryptone Soya yeast extract Broth (Merck, Darmstadt, Germany).

Composition	g/l
Tryptone	17.0
Soya peptone	3.0
Sodium	5.0
Dipotassium phosphate	2.5
Glucose	2.5
Yeast extract	6.0

Directions:

Suspend 30g of the powder in to one litter of distilled water.

Mix thoroughly.

Autoclave at 121°C for 15 minutes.

Dispense in to sterile test tubes.

G) Blood Agar Base (Becton Dickinson, Mary land, USA)

Composition	g/l
Heart muscle infusion from (solids)	2.0
Pancreatic digest of Casein	13.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	15.0

Directions:

Suspend 40g of powder in to one litter of purified water.

Mix thoroughly.

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

Autoclave at 121°C for 15 minutes (N.B do not over heat).

Cool the base to 45°C-50°Cand add 5% sterile, defibrinated sheep blood.

Dispense in to sterile petridishes.

H). SIM medium (Becton Dickinson, Mary land, USA).

Composition	g/l
Pancreatic digest of Casein	20.0
Peptic digest of animal tissue	6.1
Ferrous ammonium iron sulphate	0.2
Sodium thio sulphate	0.2
Agar	3.5

Directions:

Suspend 30g of powder in to one litter of purified water.

Mix thoroughly.

Heat with frequent agitation and boil for one minute.

Autoclave at 121°C for 15 minutes.

Dispense in to sterile test tubes.

I). **Carbohydrate** utilization broths (Rhaminose, Xylose and Mannitole).

i) Phenol red (Merck, Darmstadt, Germany).

Composition	g/l
Peptone from casein	5.0
Peptone from meat	5.0
Sodium chloride	5.0
Phenol red	0.018

Directions:

Dissolve 15g of powder in one litter of purified water.

Autoclave at 121°C for 15 minutes and cool to 60°C.

ii) Carbohydrate solutions:

Rhaminose (AES Lab., Combourg, France).

Xylose (AES Lab., Combourg, France).

Mannitol (Merck, Darmstadt, Germany).

Directions:

Dissolve 5g of each carbohydrate in 100ml of water separately.

Sterilize by filtration.

iii) Complete medium

Directions:

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

J) Ferric ammonium citrate (sigma, steinheim, Germany).

Directions:

Dissolve 5g of powder in 100ml of water separately.

Sterilize by filtration.

K). Hydrogen peroxide (3%)(sigma, steinheim, Germany)

L) CAMP test (Christie, Atkins, Munch-Peterson) test strains.

Known ß-hemolytic staphylococcus aeurus (Coagulase positive on rabbit plasma in our laboratory), Stock culture was maintained by inoculating on to TSYEA slopes incubating at 37°C for 24 hours storing in a refrigerator at 4°C. They will frequently be cultured once in a month during the whole study period.

Annex 3: Record sheet for laboratory analysis of *Listeria* species in foods on Oxford and PALCAM agars

SN	Date	Sample type	Source	Half Fraser	Fraser Broth	Growth on Oxford	Growth on PALCM	Growth on TSYEA	Gram staining	Catalase	Motility
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											
19											
20											

Annex 4: Record sheet for biochemical reactions of *Listeria* species

Sample No	Carbohydrate utilization test			Hemolysis	CAMP test with
	Rhamnose	Xylose	Mannitol		*S. aureus*
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

9. CURRICULUM VITAE

1. PERSONAL DATA

1.1. Name – Abdul Jelal Redi Hassen

1.2. Sex - Male

1.3. Date of birth – 23 sept 1968 G.C

1.4. Place of birth – Bedelle

1.5. Nationality– Ethiopian

1.6. Marital status – Married

1.7. Profession – Veterinarian

1.8. Language – Amharic – speaking and writing

- English – speaking and writing

-Oromiffa- speaking

Siltigha--speaking

1.9. Home address – Gurage Zone, Cheha woreda Agricultural Office.

-Tel (011) 3310012, (011) 3310284,

-2. EDUCATIONAL BACKGROUND

2.1. Primary Education – Ras Tesema Nadew Primary School, Bedelle

2.2. Secondary Education – Bedelle Secondary School, Bedelle

1983-1986

2.4. University Education - Addis Ababa University, Faculty of Veterinary Medicine - 1987-1992

Degree award – DVM, Degree of Doctor of Veterinary Medicine

2.5. Postgraduate Education - AAU, Faculty of Veterinary Medicine, 2008-2009

Degree award – Master of Science (MSc) in Tropical Veterinary Public Health.

EMPLOYMENT RECORD

3.1. Date of employment – 8 April, 1994G C.

3.2. Position – Veterinary services team leader and field veterinarian

3.3. Employer – Southern Ethiopian Bureau of Agr. Gurage Zone, Cheha Woreda Agricultural Office.

3.4. Major tasks – Coordinating veterinary services in Cheha Woreda

– Training of animal health assistants and technicians

4. RESEARCH ACTIVITIES

4.1. Study on the prevalence of *Listeria* species in raw milk and cottage cheese in Debrezeit, Ethiopia (MSc thesis, 2009)

4.2. Study on the prevalence and economic significance of Fasiolosis and Hydatidosis in Wolaita Sodo abattior (DVM thesis, 1992)

4.3. Review on public health importance of listeriosis. (Seminar paper, 2008)

5. REFEREE

5.1 Dr. Temima Nuri (DVM) A head of animal health Department in Gurage Zone Agricultural office.

Tel (011) 3300247

10. DECLARATION

"I declare that the information presented here in my thesis is my original work, has not been presented for a degree in any other University and that all sources of materials used for the thesis have been duly acknowledged".

Name: Abdul Jelal Redi Hassen

Signature: ____________

Date of Submission: 26 June 2009

This thesis has been submitted for examination with my approval as a University advisor.

Dr. Moses Kyule

Signature: ____________