PREVALENCE AND ANTIMICROBIAL PROFILE OF LISTERIA MONOCYTOGENES IN RETAIL MEAT AND DAIRY PRODUCTS IN ADDIS ABABA AND ITS SURROUNDING TOWNS, ETHIOPIA

By

FIREHIWOT ABERA DERRA (BSc)

Department of Microbiology, Immunology, and Parasitology,
Faculty of Medicine, ADDIS ABABA UNIVERSITY

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SCHOOL OF GRADUATE STUDIES

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Abbreviations

AAU	Addis Ababa University
AFNOR	Association Francaise de Normalization
AOAC	Association of Official Analytical Chemists
AST	Antimicrobial Susceptibility Test
ATCC	American Type Culture Collection
BLEB	Buffered *Listeria* Enrichment Broth
CAMP	Christe Atkins Munch Peterson test
CDC	Center for Disease Control and Prevention
CFSAN	Center for Food Safety and Applied Nutrition
CSF	Cerebrospinal Fluid
EHNRI	Ethiopian Health and Nutrition Research Institute
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
HACCP	Hazard Analysis Critical Control Points
IDF	International Diary Federation
ISO	International Organization for Standardization
LEB	*Listeria* Enrichment Broth
LPM	Lithium Chloride-phenylethanol-moxacalatam medium

MFA	Modified Fraser Agar
MOX	Modified Oxford Agar
NCCLS	
NCID	National Center for Infectious Diseases.
OXA	Oxford Agar
PALCAM	Polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin Mannitol
PHLS	Public Health Laboratory Service
RECC	Research and Ethical Clearance Committee
RTE	Ready to eat
TSY	Tryptose Soy Broth
UNIDO	United Nation Industrial Developmental Organization
USDA	United States Department of Agriculture
UVM	University of Vermont Listeria enrichment medium

ABSTRACT

Listeriosis is a disease of humans and animals, which is mostly caused by Listeria monocytogenes, as the result of food and environmental contamination as well as zoontic infections. In the world, this disease is becoming an emerging bacterial disease, with low incidence but high case fatality rate. The objective of this study was to isolate L. monocytogenes from retail meat and diary products (raw milk, cottage cheese, cream cake) and to detect their antimicrobial profile.

Two hundred and forty food samples were purchased from food vendors, shops and supermarkets, using cross-sectional study design, from July to December 2006. L. monocytogenes isolation were performed according to the standard procedures using Listeria enrichment broth, Modified Fraser broth, Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculine Mannitol (PALCAM) and Oxford Agar (OXA) media as well as confirmatory broths like rhamnose, xylose, mannitol; blood agar and Christe Atkins Munch Peterson (CAMP) test. The antimicrobial profile of L. monocytogenes was also assessed by using the standard disk diffusion method (Kirby Bauer, Muller Hinton agar) and it was tested against 14 antimicrobial drugs.

Out of 240 food samples tested, 66(27.5%) were positive for Listeria species. The prevalence rate of L. monocytogenes was found to be 13 (5.4%). The antimicrobial profile of L. monocytogenes was also assesed and it was found that L. monocytogenes was sensitive to most drugs except clindamycin which showed the highest resistance rate (100%) and also to certain extent, to chloramphenicol (53.9%), tetracycline (31.8%) penicillin (23.1%), and rifampicin (15.4%).

It was shown that L. monocytogenes is prevalent in Addis Ababa and other small towns which indicated that it might also be present in other towns of the country with similar prevalence, which requires a surveillance system.

Key words: Listeriosis, Prevalence, Antimicrobial susceptibility

CHAPTER 1: INTRODUCTION

Listeria monocytogenes is a member of Genus *Listeria* which is a Gram-positive, cocco-bacillus and it is a facultative anaerobe, non-spore forming bacterium which measures 0.4 µm in width and 1 to 1.5 µm in length. The bacterium is motile being flagellated, especially below 33^0C via 1-5 peritrichous flagella (Fig 1) and grows with optimum temperature between 30-37^0C. It is catalase positive and can grow at temperature of 0 to 5 ^{0}C (Jennifer *et al.*, 1980; Cox *et al.*, 1989).

Since it is morphologically similar to *Erysipelothrix* and diptheroides, it was earlier classified within the family Corynebacteriaceae. However, its true taxonomic position is now agreed to be within a Clostridium sub-branch, which is confirmed by 16SrRNA (Ribosomal Ribonucleic acid) partial sequencing. The genus *Brochothrix* is phylogenetically closest to *Listeria.* (Vázquez-Boland *et al*., 2001).

Listeria comprises about six to eight species, (Harvey and Gilmour, 1992; FDA/CFSAN, 2003; USDA, 2005). However, it is presently accepted that there are six species of the genus, but it continuously undergoes constitutional changes. The known species are *L. monocytogenes*, a human and both wild and domestic animal pathogen, being ubiquitous, in the environment, including in any food chain and the etiological agent of listeriosis; *L. ivanovii*; a pathogen to animals (mainly sheep), and causes some complications like abortion; *L. innocua*; which is the species most frequently found in the environment (85%) (Molla *et.al*., 2004).

The remaining species, *L. welshimeri, L. seeligeri, L. grayi* and *L. murrrayi* are mostly found in animals and in the environment, but they are mostly reported as non-pathogenic organisms. The latter two and *L. denitrificans*, (which is not mentioned up here), have difficult taxonomic classification, not well known (Uncertain). Some literatures suggested that, *L. grayi* had subspecies which comprises of *L. grayi* subspecies *grayi* and *L. grayi* subspecies *murrayi.* Similarly, *L. ivanovii* has two subspecies, *L.ivanovii* subspecies *ivanovii* and *L. ivanovii* subspecies *londiniensis* (FDA/CFSAN, 2003).

The major pathogenic species in this genus is *L. monocytogenes*, which was first described by Murray and Pirie while they were working in Cambridge and Johannesburg in 1926 and 1927 respectively. It was cited by Ryser and Donnelly, (2001) and they reported that, the two scientists independently isolated the same organism, but gave it different names. Murray isolated the organism from septicemic rabbits and named it *Bacterium monocytogenes*, because the organism increased monocyte production in the host. A year later, Pirie isolated this bacterium from wild Gebrils, and called it *Listerella hepatolytica*, after Joseph *Lister*, a pioneer of antiseptic surgery in the 1860's (Vázquez-Boland *et al*., 2001). However, the species name *Listeria monocytogenes* was agreed up on in 1940. Then after, the other *Listeria s*pecies were discovered consecutively. These are *L. innocua* in 1979, *L. welshimeri* in 1983, after H.J.Welshimer (American bacteriologist), *L. seeligeri* in 1983 by German bacteriologist H.P.Seeliger, *L. ivanovii* in 1984, by Buligarian microbiologist, *L. murrayi* in 1971, and *L. grayi* in 1966 by American bacteriologist, M. L. Gray (Ryser and Donnelly, 2001; Vázquez-Boland *et al*., 2001).

The genomes of both *L. monocytogenes* and *L. innocua* have been sequenced. The guanosine-cytosine (G+C) content of their DNA is reported between 36-38% (Jemmi and Stephan 2006). *L. monocytogenes* has a single circular chromosome, while *L. innocua* also contains a plasmid of 81,905 base pairs (bp). The genome of *L. monocytogenes* strain EGD (serotype 1/2a) is 2,944,528 bp long with 2,853 open reading frames and a G + C content of 39%. The genome of *L. innocua* is 3,011,209 bp long with 2,973 open reading frames and a G + C content

of 37%. Surprisingly, many encoded proteins are similar to those of the soil bacterium *Bacillus subtilis*. (Jemmi and Stephan, 2006)

Human listeriosis is commonly associated with *L. monocytogenes*. However, *L. ivanovii* and *L. seeligeri* can also be a cause of listeriosis but extremely in rare cases (Vázquez-Boland *et al*., 2001).

A number of food borne outbreaks caused by *L monocytogenes*, have so far been reported, which were known to cause serial deaths in a number of individuals and in different regions, especially in Europe and the USA (Todar's, 2003). However, in most African countries, there are a few reports on *Listeria* and listeriosis, when compared to the Europe and USA (Molla *et al*., 2004). This is because; the organism has not been given much attention, may be due to lack of adequate facility, life style differences and RTE foods are more common in USA and Europe than in Africa.

Hence, the main objective of this project was to isolate *Listeria monocytogenes* in some selected food products, using standard bacteriological parameters and to determine the distribution of the species in those indicated 12 regions of Ethiopia.

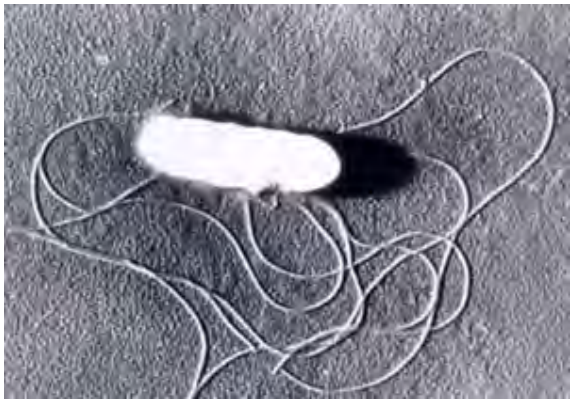

Fig..1: *Listeria monocytogenes* Scanning EM showing pertirichous Flagella
Adapted from Todar's Online of Textbook of Bacteriology (Todar's online, 2003)

CHAPTER 2: LITERATURE REVIEW

2.1. Epidemiology

Listeria species are found worldwide and everywhere, in animals, in foods, in humans, in soil, in the food processing environment, in contact surfaces and also in food containers (Peeler and Bunning, 1994; FDA/ CFSAN, 2003). Until about 1960 it was associated almost exclusively with infections in animals and less frequently in humans. However, in the last 30 years it has been known that it was widely distributed in nature (Vázquez-Boland *et al*., 2001).

Of the several species of *Listeria*, *L. monocytogenes* is an important cause of wide spectrum of diseases in animals and humans. Species of *Listeria* have been isolated from at least 37 species of mammals (some literatures suggested about 42 wild and domestic mammals), 17 species of birds, and from others like flies, ticks, fish, crustaceans, and oysters (Opinions, 1999; Todar's , 2003).

Nowadays, *L. monocytogenes* is becoming an emerging food borne pathogen in the world. Earlier it was known to occur predominantly, in most European and Western countries. Large number of gastroenteritis cases due to this organism, have been reported mainly from USA (Todar's, 2003).

L. monocytogenes is detected in different types of food and its prevalence rate differs from place to place, based on the hygiene, food content and environmental contamination rates. It was reported by several workers and also by the European commission to be found in raw milk 2-12 % (Peeler and Bunning 1994), in pasteurized milk and milk products, 2-30 % (Buchannan *et al*., 1997; Molla *et al.*, 2004; Sarkar, 2006), in fresh meat 0-68% (Jennifer *et al.*,1980; Opinions 1999), in minced beef 7-36%, in meat products 0-52%, (Yang et al., 2006), in poultry 0-85%, in fish products 4-60% (Opinions 1999), in vegetables, salads 1-12%, (Sagoo *et al*., 2003), in Ice cream 22%, and about 9% in ready to eat foods (Opinions,1999; Richards, 2000). In addition, it was also detected in other foods like lettuce (Little *et al.*,1999), cold smoked fish (Mel et al., 1994), in diary products (Pritchard et al., 1994), in vacuum processed meat (Grau and Paul, 1992; Vermairen *et al.*, 2000) and even on the hands of food processors (Kerr *et al.*, 1993), which showed that this organism to be found everywhere and affects many animals or humans.

In another study, Girdhar and Garg (2002) reported that the carriage rate of *Listeria* in animal farms and showed that silage had the maximum prevalence rate of the organism (75%), followed by fodder (43.5%), feed (33.5%), fecal matter (20.5%), sewage (16.6%) and soil (4.8%). It was also isolated from 2-6% of fecal samples from healthy people (Rocurrt & Cossart, 1997), which shows, humans and animals can be asymptomatic excretors, thus introducing *L. monocytogenes* into the environment (Uyttendael *et al*., 1997).

On the other hand, a research done on a single cow milk sample showed that the cow was shedding between 2000- 20,000 *L. monocytogenes* cells/ml. It is estimated that animal farms worldwide have about 2% cows harboring *L. monocytogenes* (Peeler and Bunning, 1994; Pritchard *et al.*, 1994).

For some pathogenic bacteria, the minimum infectious dose is well established, but there is no certain figure for *L. monocytogenes* despite the considerable research undertaken since 1985 (Uyttendael *et al*., 1997), but it was estimated in the analysis of food samples implicated in outbreak as between $3X10^1$ to $1,6X10^9$ CFU/gram with median of 10^5 (Maijala, *et al.*, 2001).

The incidence of listeriosis in humans is very low, in the order of 3-8 cases per million inhabitants per year in the industrialized countries (Johan *et al.*, 2004*)*. Most countries within the EU have an annual incidence between 2-10 reported cases per million populations per year (Valk *et al.,* 2005). However, because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food borne illnesses: it ranks second, after salmonellosis, in the USA and France; and fourth in England and Wales (Opinions, 1999). The number of new illness cases, in comparison with other food borne pathogens, per year per 100,000 population in the EU and the USA, respectively, was reported recently as, *Salmonella* (42 vs. 14.5), *Campylobacter* (48 vs. 20), *Listeria* (48 vs. 20), E. coli (1.3 vs. 0.9), and *Yersinia* (2.4 vs. 3.9) (Gutler, 2006).

Even though, the incidence of human listeriosis is low about (2-10/million) as indicated above, the case fatality rate is reported to be, between 20 and 40% in the United Kingdom (UK), and around 20% in the USA. This percentage can increase up to 75% in immuno-compromised individuals (Opinion, 1999), which are one of the major risk groups. For example, people with Human Immunodeficiency Virus (HIV) are almost 300 times more likely to get listeriosis than other healthy people. In addition, pregnant women are about 20 times more at risk than others and about 1/3 of listeriosis cases occur during pregnancy, late in 2^{nd} or in 3^{rd} trimester or 3 weeks of the newborn life. Moreover, about 370 unborn or newborn children are infected annually in the United States. Of these, 80 die and 56 suffer with lifelong complications (*www.bfhd.wa.gov, 2003*).

Due to *L. monocytogenes*, a number of major outbreaks of human listeriosis have been reported (Morrow *et al.*, 2004). The first confirmed food-borne outbreak of listeriosis occurred in 1981 in Nova Scotia, Canada, and involved 41 patients. Probably the epidemic was due to sheep manure cultivated cabbage (Opinions, 1999). Another outbreak that involved over 100 people was reported. Of these 34 occurred in pregnant women, among whom there were nine still births, 23 infants born infected and two live healthy births. There was also 30% mortality among 77 non pregnant adults (Todar's, 2003).

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

Mostly, the source of such major outbreaks was reported to be dairy products (Kerr *et al*.,1993), which were unpasteurized milk, Mexican style soft cheese and Swiss regional type soft cheese (Kerr *et al*., 1993; Molla *et al*., 2004). The outbreak of the Mexican style cheese associated listerial infection, occurred in California that resulted in 142 cases and 48 deaths (Kerr *et al*., 1993).

In Europe, between 1991 and 2002, a total of 19 outbreaks of invasive listeriosis were reported in nine different countries, with a total of 526 outbreak related cases while the number of reported outbreaks increased gradually over time, from seven outbreaks detected in the period 1992-1996 to 11 in 1997-2001. In addition, four outbreaks of acute listerial gastroenteritis were reported: Of these, Italy had two outbreaks in 1993 (18 cases) and in 1997 (1566 cases); and one was in Denmark in 1996 (3 cases); and the fourth was in Belgium in 2001 (2 cases of acute gastroenteritis and one case of invasive Listeriosis) (Valk *et al.*, 2005). The mean incubation period of non-invasive gastroenteritis, which occurred in 1997, in Italy was reported to be 1-2 days, with diarrhea lasting from 1-3 days. The microorganism has gained recognition as food borne bacterium because of its association with epidemic gastroenteritis (Morrow *et al*., 2002).

In Australia, there were also two reported outbreaks. In 1990 and 1991, six women gave still births and three ill people were reported, respectively. Laboratory tests confirmed the presence of *L. monocytogenes* (10^7 cells /gram) after checking samples of mussels that was eaten by the infected people. Similarly, in 1992, there were 270 reported cases of listeriosis in France that resulted in 63 deaths and 22 abortions, after eating pork tongue in jelly (FDA/CDC, 2003).

CDC (Center for Disease Control and Prevention) (2000) reported that, about 2500 illnesses have annually occurred due to *L. monocytogenes*, with higher incidence rates during the summer season. Of these, 500 were fatal cases. In the similar manner, at 2001, 94 critical cases were found (FDA/CDC, 2003).

In developing countries like Africa and Latin America, most of the times, there have been few or no reports on *L. monocytogenes* (Molla *et al.,* 2004). This might be true because no one has given it due attention. or were unaware of its occurrence. However, nowadays there are some reports on prevalence of *L. monocytogenes* in different samples. For example, in Nigeria, occurrence of *L. monocytogenes* in environmental samples and vegetables were studied and reported as 85% from cow dung, 91.6% from soil and 73.75% from vegetables (David & Odeyemi, 2007). In Ethiopia, there was also a work done, in 2004, which showed a prevalence rate of *Listeria* species about 31.6% and *L. monocytogenes* 5.1% in some foods, such as, meat, cheese, fish, pork, poultry, ice-cream (Molla *et al.*, 2004).

As a result of such environmental contaminations and poor sanitary conditions while handling of the food samples, during harvesting, processing, packing, preparing or cooking times; many people could be infected and would have listeriosis at any time.

L. monocytogenes is transmitted from man to man or from animal to man mostly through ingestion of the organism with contaminated food. It was reported that, approximately 99% of human listerioisis appear to be food-borne. The contamination sources are animal or human fecal matters. However, animal hides are found to be the most important source of contamination than fecal matter (Jennifer *et al*., 1980).

After entry following ingestion, the organism has a long incubation period, 1-90 days. In immuno-competent individual, listeriosis may occur mostly after being exposed to higher doses of the organisms. However, exposure of vulnerable individuals to even low doses may end up in acquiring listeriosis (Todar's, 2003). The clinical course of infection usually begins about 20 hours after the ingestion of heavily contaminated food in case of gastroenteritis, whereas the incubation period for the invasive illness is

around 20 to 30 days or much longer. Similar incubation period have been reported in animals for both gastroenteritis and invasive disease (Vazquez-Boland *et al*., 2001)

The common risk factor for *Listeria* infection are food storing time, temperature, type of product, infective dose, immunity, and traditionally accepted consumption of raw foods. The following factors might contribute to the occurrence of high incidence of listeriosis in the future (Rohrbach *et al*., 1992); the increased proportion of susceptible people due to age, immuno-compromised diseases or treatment; increased use of cold storage to prolong the shelf life of foods; consuming of raw foods like raw milk or cheese from unpasteurized milk etc, especially which are known to harbor dangerous pathogens (Oliver *et al.,* 2005).

For example, *L. monocytogenes* has been reported several times from raw milk, in different countries; in USA, in 1987 (4.2%), in 1992 (4.1%), in 1997 (4.6%); in Canada, in 1988 (1.3%, and 5.4%), and in 1998 (2.7%); in South Africa, in 1990 (5.2%); in Ireland, in 1992 (4.9%) and in England and Wales (5.1%). Surprisingly, the center for science in the public interest of US, presented that, 28 states currently allow the sale of raw milk to the community at the company level. Whereas the dairy farmers of Canada discussed historical overview of Canadian raw milk laws and made it clear that, sales of raw milk are illegal across Canada and can lead to fines of up to $250,000. Quebec is the only province that allows for raw milk cheese production. Such similar trend like the Canadian law has to be stated everywhere in order to protect the consumers, from such devastating bacterial pathogens (Chapman, 2006).

2.2. Pathogenesis, Pathology and Virulence factors

Following ingestion of the bacteria with contaminated food, it reaches the digestive tract and start secreting invasin /Internalin (In1A & In1B), which enables the *Listeria* to penetrate the host non-phagocytic cells of the epithelial lining and binds with extracellular domain of E-cadherin (a transmembrane cell to cell adhesion molecule) and then disseminate into circulatory system to cause systemic diseases. The anatomical site and the mechanism by which *L. monocytogenes* breaches the intestinal barrier are controversial. In mice, *L. monocytogenes* invasion and replication within

the gastrointestinal tract is independent of InlA and is restricted to the peyer's patches, suggesting a predominant role for specialized phagocytic M-cells in bacterial uptake. In similar manner, in rats, intestinal translocation rates are low and independent of the inlAB locus, suggesting a passive process that does not involve InlA or InlB. However, mice and rats are not natural hosts and further more, mouse and rat E-cadherin differ from human E-cadherin at an important amino acid residue that renders cells resistant to InlA-mediated invasion. In contrast to this, *L. monocytogenes* can directly invade enterocytes in guinea pigs, which are naturally susceptible to listeriosis. In transgenic mice, enterocytes expressing human E-cadherin are also susceptible to invasion by *L. monocytogenes* (Vazquez-Boland *et al*., 2001)

L. monocytogenes is capable of multiplying extracellularly and intracellularly, within macrophage after phagocytosis or within parenchymal cells after induced phagocytosis, and then spread to hepatocytes of the liver, causing cell disruption. It is internalized by phagocytic and non-phagocytic cells and can deliver antigen to both endogenous and exogenous antigen processing and presentation pathways. *Listeria* latch onto receptors on the host cells and are then engulfed and remain as a membrane-bound vesicle, but *Listeria* soon escapes into the cytosol (Rocourt & Cossart, 1997). It is also able to penetrate the endothelial layer of the placenta and thereby infect the fetus (Todar, 2003). However, this will occur only if the immune system of the individual is compromised. This is because normally the immune system eliminates the organism before it spreads by producing T-lymphocytes.

Since *L. monocytogenes* multiplies intracellularly, the effective host response is cell mediated immunity, which involves both TH1 (CD4+) cells and Tc (CD8+) cells that stimulates production of TNF (Tissue Necrotizing Factor), gamma interferon, macrophage activating factors and a cytotoxic T cell response (Todar, 2003; Opinions 1999). In general, protection against *Listeria* is mediated via lymphokine activation of T cells on macrophages and by interleukin-18 (Vazquez-Boland *et al*., 2001).

Majority of *L. monocytogenes* strains are virulent (Opinion, 1999), by having different peculiar properties. For instance, they are able to multiply at low temperature, so that they multiply and accumulate in such refrigerator stored foods; and also are actively motile by means of pertrichous flagella at room temperature (20-25^{0}C), which is

helpful for existence and spread in the environment. Surprisingly, they don't synthesize flagella at body temperature. Therefore in this case, the virulence is associated with the ability of the bacteria to move themselves into, within and between host cells, by polymerization of host cells actin (gene product Act A) at one end of the bacterium, that can propel the bacteria through cytoplasm (growing actin tails) and, helps for movement, attachment, penetration and cell to cell spread following phagocytosis and engulfment of such infected cells by adjacent cells. (Fig 2). Recently, evidence has been presented that ActA may also be involved in the entry of *L. monocytogenes* into eukaryotic cells, probably by recognition of an HSPG (Heparan Sulfate Proteoglycan) receptor (Vazquez-Boland *et al*., 2001). In addition to this, they have the properties of adherence and invasion as other virulence factors. *Listeria s*pp. InlA and InlB, are proteins encoded by the inlA and inlB genes. InlA was shown to function as an invasin, mediating bacterial internalization by these normally non-phagocytic epithelial cells (Gaillard *et al.,* 1991; Dramsi *et al*., 1995). The organism is thought to express D-galactose residues on its surface, so able to attach to mannose binding receptors on the host cells. Macrophages are well known to express this receptor, such that they simply take up, the organism by induced phagocytosis. However, following this reaction the organism is able to escape from the phagosome before phagolysosome fusion occurs by releasing its toxin product called Listeroiolysin (LLO), which acts as haemolysin (hly gene) of other Gram-positive bacterial secretions e.g. Streptolysin O (Jenkins *et al*., 1964; Kingdom and Sword, 1970; Geoffroy *et al*., 1987).

They also have other virulence factors which includes those secreted as two other types of haemolysins; phosphatidyl inositol specific phospholipase C (Pl-Plc) and phosphatidyl choline-specific phospholipase C (Pc-Plc), by which it can easily disrupt membrane lipids. Two, PlcA and PlcB, are present in *L. monocytogenes* and *L. ivanovii*; the third, SmcL, is specific to *L. ivanovi* similar to that responsible for the lecithinase activities of *Bacillus cereus* and *C. perfringens* (Vazquez-Boland *et al*., 2001). Phospholipase is required for efficient lysis of the secondary phagosomes formed after listerial cell-to-cell spread (Marquis *et al*., 1995).

In addition to these virulence factors, other listerial products have been identified that also contribute to survival within the host such as p60 (iap), antioxidant factors, metal

ion uptake and stress response mediators (Vazquez-Boland *et al*., 2001). It produces catalase and superoxide dismutase, which enables the organism to neutralize the effects of phagocytic oxidative burst. In addition, it produces a Zn++ dependent protease, which is thought to act as some exotoxin and has imaBA gene which encodes a 20Kda protein (LmaA), that induces delayed type hypersensitivity and other Cell mediated immunity responses (Todar's, 2003).

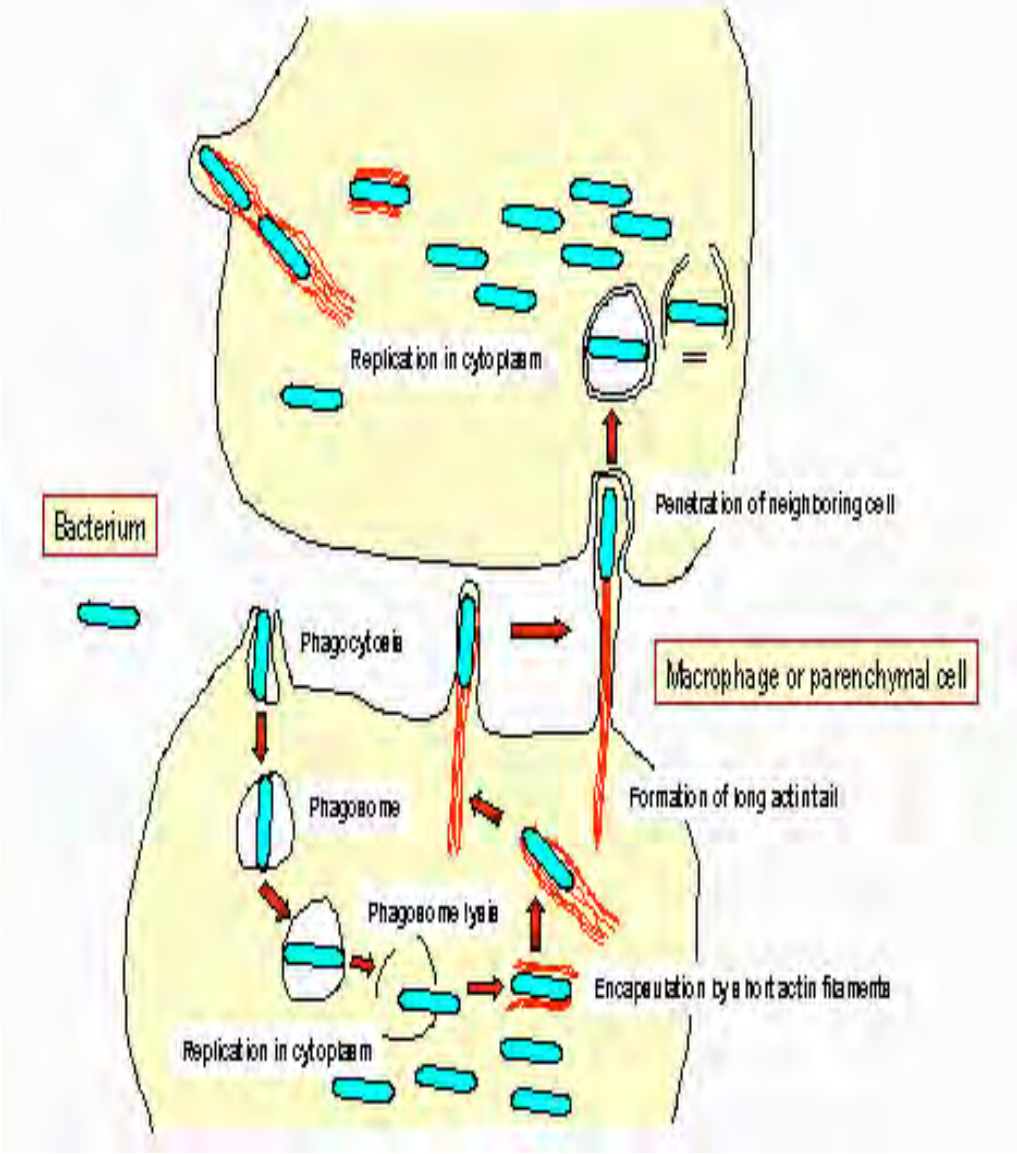

Fig 2. Cell to cell spread of *L. monocytogenes* by formation of actin filaments (act A gene)

Adapted from Todar's online textbook of Bacteriology (Todar's, 2003)

L. monocytogenes causes both perinatal and adult listeriosis in humans. Perinatal human listeriosis may cause intrauterine infection resulting in intrauterine sepsis and death before or after delivery. Adults can develop meningo-encephalitis, bactermia and some times focal infections.

Listeriosis is a disease, which encompasses a wide variety of disease symptoms. For example, in ruminant animals, the most common form of the disease is encephalitis while in young animals, visceral or septicemic infections as well as meningitis do often occur. In cattle and sheep intrauterine infection of the placenta of the fetus frequently results in abortion (Vazquez-Boland *et al*.., 2001).

While in a normal immuno-competent individual the infection is usually asymptomatic or with mild influenza-like symptoms, it can be carried with or without apparent symptoms, up to 5-10% in the intestinal tract of humans. However, in susceptible individuals like; younger, older, pregnant women and immuno-compromised (YOPI) individuals (people with Cancer, HIV, rheumatoid disease, diabetes, cirrhosis of the liver, asthma), illness is likely to occur with serious (overt) form of the disease. In Europe, North America, Japan and Australia the proportion of elderly people (>65years of age) expected to double with the next 30-35 years and in countries like Germany and Italy this proportion approaches to about 30% (Opinion, 1999; *www.bfhd.wa.gov*, *2003*).

The clinical manifestation in the overt form of the disease is mainly meningitis, septicemia or meningitis with septicemia, skin lesion/ rash, CNS symptoms (headache, stiff neck, confusion, loss of balance) and food poisoning (nausea, vomiting, diarrhea, fever, chills muscle ache) and also focal infections (abscesses, septic arthritis, osteomyelitis, endocarditis, etc.). Apparently healthy individuals may also be affected (Letter, 1989; Opinion 1999).

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

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

Listeria meningitis is mainly characterized by an acute form of the disease with seizures and ataxia, blood cultures are positive in 75% of cases. *Listeria* demonstrates "tumbling motility" in wet mounts of cerebrospinal fluid (CSF). Gram stain of CSF may be negative in up to 60% of cases and CSF glucose is normal up to 60% of cases and also mononuclear cells in the CSF predominate in one-third of cases. In addition, CSF protein levels are moderately elevated and CSF culture findings are positive in nearly 100% of patients' whereas, serological testing is not reliable. Stool cultures are neither sensitive nor specific in this case. In related to this, other CNS infection like brain abscess and endocardities are diagnosed by using either CT scan for brain stem and echocardiography for endocardities are indicated (Vazquez-Boland *et al*., 2001). Another frequent form of listeriosis is bacterimia or septicemia (15 to 50% of cases) with a high mortality rate (up to 70%) in debilitating conditions.

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

In pregnant women infection of the fetus is extremely common and can lead to abortion, still birth or delivery of *Listeria* infected infant. Granulomatosis infantiseptica is one of the syndromes, which is associated with feto-maternal or neonatal form of meningitis and is characterized by the presence of pyo-granulomatous abscesses disseminated over the body. The neonatal form of infection is reclassified as early- onset of sepsis, which results in premature birth and late- onset

meningitis, which is acquired through vaginal transmission. Of all pregnancy-related cases, 22% result in fetal loss or neonatal death, but mothers usually survive. Corticosteroid therapy is the most important predisposing factor in patients who are not pregnant. CNS infection in general, is very rare during pregnancy, although it is observed frequently in other compromised hosts. For example, brain abscess occurs in 10% of CNS infections, often located in the thalamus, pons, and medulla. This unusual complication is associated with high mortality (Vazquez-Boland *et al*., 2001)

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

2.3. Laboratory isolation methods

2.3.1. Culture method

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

evidenced by blackening of the medium. In addition, different antimicrobial agents like acriflavin, nalidixic acid, cyclohexamide are supplemented with those media to suppress the growth of other non-*Listeria* species of bacteria and other microbial agents (see Annex II).

Various conventional methods are known for the isolation of *L. monocytogenes* and other *Listeria* spp. from food and clinical samples that have gained acceptance for international regulatory purposes. All the methods differ from each other by their criteria of selecting primary, secondary and also selective agars (inhibitory compounds) in regard to the food types. For this project IDF (International Dairy Federation) method was used. In association with this, the tolerance limit of *L. monocytogenes* differs from country to country and also based on the food type. For instance, the USA requires absence of *L. monocytogenes* in 25 g of ready to eat foods (Zero tolerant) while some European countries have a tolerance level of 100 CFU/ml of *L. monocytogenes*/ml (FDA/ CDC, 2003).

Table 1. The following table shows the types of the methods and their choice of media (Manual, 2004)

Type of the method	1^0 selective enrichment used	2^0 selective enrichment used	Selective media
The United States Food and Drug Administration, Center for Food Safety and Applied Nutrition (FDA-CFSAN) method,	*Listeria* enrichment broth (LEB)	Non/ LEB II	lithium chloride-phenylethanol-moxalactam medium (LPM) and Oxford agar plates
The Association of Official Analytical Chemists (AOAC) official method,	Trypticase Soy broth with 0.6% yeast extrract	-	PALCAM
The International Organization for Standardization (ISO 11290) standard,	half Fraser broth	Fraser broth	PALCAM and Oxford agar
The International Dairy Federation (IDF) method,	Modified *Listeria* enrichment both (LEB)	Either non/Fraser broth	PALCAM and/or Oxford
The United States Department of Agriculture (USDA) – Food Safety and Inspection Service (FSIS)	University of Vermont medium	either UVM II or Fraser	lithium chloride-phenylethanol-moxalactam

method,	(UVM I)	broth	medium (LPM)
The French Standards (Association Francaise de Normalization, AFNOR) method	Demi-Frase broth	Fraser broth	Oxford or PALCAM
Health Canada method for foods and environmental samples	*Listeria* enrichment broth (LEB)	Modified Fraser broth	Oxford agar, lithium chloride-phenylethanol-moxalactam (LPM), modified Oxford or PALCAM agar

2.3.2 Biochemical test

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

Table 2. Biochemical chart for *Listeria* species identification;

Species	Production of acid			Hemol -ysis	CAMP test	
	Xylose	Rhamnose	Mannitol		*S.aureus*	*R.equi*
L. monocytogenes	-	+	-	+	+	-
L .innocua	-	+/-	-	-	-	-
L .ivanovii	+	-	-	++	-	+
L. seeligeri	+	-	-	(+)	(+)	-
L. welshimeri	+	+/-	-	-	-	_
L.grayi subsp. Grayi	-	-	+	-	-	-
L. grayi subsp	-	+/-	+	-	-	-

Murrayi						

+/-: variable; (+): weak reaction; +: 90% positive reaction; -: no reaction (-). R. equi; *Rhodoccocus equi* (FDA/CFSAN, 2003; Jemmi and Stephan, 2006)

2.3.3 Chromogenic/Rapid test

Rapid detection methods are available by using several methods such as PCR (Polymerase chain reaction), monoclonal antibodies and nucleic acid hybridization tests, and fluorogenic tests. In addition, alternative new chromogenic differential selective agars like BCM, ALOA, CHROM agar *Listeria* and Rapid L.mono can be used in parallel with one of the selective agars (Cox *et al.*, 1998). FDA recommended and validated such chromogenic rapid kits with threshold of detection greater than 10^4 CFU/ml of enrichment culture and incubation period of 4-6 hours. The advantage of such tests, are their principle lies on identifying of a specific virulence factors like phosphatidylinositlol specific phospolipase and in enumeration of the organism in the food samples (FDA/CFSAN, 2003).

2.3.4 Serological test;

If possible, all culture isolates of *Listeria* species should be typed serologically and genetically according to FDA rules (FDA/CFSAN, 2003), but it is expensive. Such serotyping is performed by mixing commercial sera and *Listeria* species suspending in fluorescent antibody buffer, after several washing and pelleting steps, in order to observe the positive clotting/precipitating reactions. Usually serological classification is done only in reference laboratories and is primarily used for epidemiological studies. These methods target to detect flagella or somatic Sub-factors. Most *Listeria* species have different serotypes in which they share one or more antigens with *L. monocytogenes*. The following serotypes are exhibited within the species and also known as serotypes of *L. monocytogenes*, these are 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Of these 1/2a, 1/2b, and 4b are mostly isolated from patients and environment that account for 92% of the isolates among humans and

animals and 4b is associated with cheese made from unpasturized milk (Doumith *et al*., 2004).

There is a significant geographical variation and 4b is identified as the predominant serotype in Canada, USA and Europe. Fifty percent of the sporadic infection outbreaks have been determined as serotype 4b.

2.4. Antimicrobial susceptibility pattern

Many antibacterial drugs inhibit *Listeria* in vitro. Some literatures reported that, *L. monocytogenes* is susceptible to wide range of antibiotics like ampicillin, erythromycin, kanamycin, streptomycin, nalidixic acid and others except for cephalosporin and fosomycin/ fluoroquinolones (Abelardo et al., 2001; Williams *et al*., 2005: Hansen *et al*., 2005; Aarestrup et al., 2007). However, it has been reported that resistant *L. monocytogenes* strains were found frequently from time to time, even to common conventional drugs like clindamycin, sulfomethoxazole (Shen *et al*., 2006) and also to enrofloxacin (Antunes et al., 2002). Plasmid-borne resistance to chloramphenicol, macrolides, and tetracycline has also been identified (FDA/CFSAN, 2003).

Combination (synergy) testing of different antimicrobial drugs against *L. monocytogenes* has also been done, which showed most effective killing power (MacGowan *et al*., 1990). The first choice of therapy is ampicillin combined with different aminoglycosides like gentamicin, TMP/SMX is recommended as the second choice of therapy or as alternative agent. However, other agents are mostly reported as static, and not cidal. On the other hand, the antimicrobial product activity of some aquatic bacteria, were also tested, which showed good inhibitory ability (Motta *et al*., 2004).

2.5. Treatment and Prevention

L. monocytogenes is a poor competitor (Desse and Taye, 2001) that does not grow in the presence of high competitor organisms like, Lactic Acid Bacteria (LAB). Some scientists use this principle for inhibiting its growth from different food samples.

Listeriosis can be treated by using conventional antibiotics like ampicillin and penicillin following diagnosis. However, the antimicrobial profile of the organism at particular place, like within the country, and at the individual level has to be known to be effective in treating patients and in reducing blind antimicrobial treatment, which may lead to the emergence of antimicrobial drug resistant strains of *L. monocytogenes.*

Antibiotic therapy is the treatment of choice in most of the complications and the dose and duration of the treatment differ accordingly. For instance, bacteriamia should be treated for 2 weeks, if the patient is immuno-competent. However, longer courses may be required in the immuno-compromised patient. Similarly, meningitis should be treated for 3 weeks; while endocarditis for 4-6 weeks; and brain abscess for a minimum of 6 weeks. Ampicillin without or with gentamycin is generally considered as the preferred agent but other effective agents like co-trimaxazole can be used for empirical antimicrobial treatment (Beek *et al*., 2002; Hansen *et al*., 2005).

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

L. monocytogenes can survive and multiply within the range of 1°C to 45°C. There are large strain-to-strain variations but some strains seem to be able to grow down to about –1.5°C. The relationship between temperature and *L. monocytogenes* rate of multiplication was studied and reported that the colder the temperature, the slower rate of multiplication was observed (Johan *et al*., 2004*).*

Growth can occur in the presence and almost absence of oxygen, and some strains may grow in foods with a pH down to 4.5 and a water activity (aw) down to 0.9. In common with other non-sporing Gram-positive pathogens, *Listeria* is readily destroyed by heat (pasteurization: 72 to 75°C at the coldest point) (Doyle, 1999a).

Table 3. Limits for growth of *L. monocytogenes*

	Minimum	Optimum	Maximum
Temperature(0C)	-0.4	37	45
pH	4.39/(4.1)	7.0	9.4/9.6
NaCl			10%
Water activity	0.9 (Glycerol,30^0C)		
Water activity	0.92 (NaCl)		
Water activity	0.93 (Sucrose)		

Adapted from: Opinions of the scientific committee on Veterinary measures relating to public health on L. monocytogenes. (Opinion, 1999)

Several researchers have noted that, different organic acids like acetic acid, lactic acid, citric acid and malic acid play a major role for inhibition of *Listeria monocytogenes* in culture media. For example, acetic acid has more potent anti listerial effects than lactic acid/HCl, and causes greater cell destruction when applied in culture media (Doylle, 1999a). Infact it is dependent on the status of the acid whether the acid is total or undissociated. Another good example here is sodium diacetate, which inhibits the growth of *L. monocytogenes* in broth cultures. It was proved that a dip in 5% acetic or lactic acids of some edible foods, not only killed *L. monocytogenes* but prevented its growth during 90 days of storage (Doylle, 1999a) Similarly, it was confirmed that, lactate and diacetate are effective against *L. monocytogenes* contamination in cured ready-to-eat meat products, and also reported about effectiveness of anti-mycotic agents such as benzoate, sorbate, pediocin and propionate in combination with nitrite in inhibiting listerial growth in ready-to-eat meat and poultry products (Katla *et al*., 2003). The growth of *L. monocytogenes* can be prevented, or at least reduced, by other means, for example by reducing the pH or by increasing the salt content. (Morrow *et al*., 2004)

On the other hand, *L. monocytogenes* is also proved to be sensitive to high pressure treatment of 400-500Mpa in order to achieve a useful kill rate from food samples or culture media (Doylle, 1999b).

2. 6. Significance of the study

L. monocytogenes is ubiquitous in nature and able to grow at low temperature, under aerobic, micro aerophilic, anaerobic condition and even under vacuum (Opinion's 2005, Desse and Taye, 2001). It is also recovered from dead vegetation and from feces of animals, which may serve as a source of contamination of agricultural crops

(Petran *et al.*, 1988). In addition, it has the ability to survive in the manufacture and ripening of different types of cheese, or meat, regardless of treatments such as freezing and surface dehydration. Its doubling time in refrigerator is one and half days. In addition to this, it is more heat resistant and less sensitive to food preservative like salt, nitrates and smoke. Moreover, it forms biofilms, which are highly resistant to antibiotics (USDA, 2005). It was investigated in culture biofilms and demonstrated that *L. monocytogenes* produced biofilms with greater biovolume and mean thickness in mixed culture than in monoculture like they are forming, in any surface area of food processing plants (Neetoo, 2006; Gilbert *et al*., 2004). In agreement with this concept, in another study, the growth of *Listeria monocytogenes* in soil and on radishes grown in contaminated soil was reported that, *L. monocytogenes* was found to be able to survive in soil for up to four weeks and colonize mature radishes and could not easily be removed from the radish surface (Danyluk, 2006).

In association with this peculiar feature of the organism, it might be one of the etiological agents of meningitis, abortions, in our country although such study has never been made so far. Therefore this project will be very helpful, as a base line data; by providing additional information on this organism and its distribution in Addis Ababa especially in relation to our culture which favors raw food eating habits and by studying its antimicrobial profile, appropriate drugs will be suggested, for treating *L. monocytogenes* associated diseases.

It will serve also as a base-line data of the prevalence of *L. monocytogenes*, in food products to be used for future research work designed in large scale as well as for surveillance of food-borne diseases caused by these pathogens in the Ethiopian community.

CHAPTER 3: OBJECTIVES

3.1. General objective;

- To isolate *Listeria monocytogenes* from raw meat and dairy products (raw milk, cottage cheese, cream cake) and to determine its antimicrobial susceptibility pattern.

3.2. Specific objectives;

- To determine the prevalence of *Listeria monocytogenes* in retail meat and dairy products
- To determine the drug sensitivity pattern of *L. monocytogenes* isolates in Addis Ababa and its surrounding towns of Ethiopia.
- To assess the presence of *Listeria* species other than *Listeria monocytogenes*
- To suggest some preventive methods against *Listeria*

CHAPTER 4: MATERIALS AND METHODS

4.1. Study Design and Area

A cross sectional study was conducted between August 2006 - December 2006, at the Public Health Food microbiology laboratory of Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia. This laboratory serves in analyzing different specimen including edible food samples like, meat and meat products; milk and milk products, vegetables, cereals, fish, beverages and other consumable products. It has been performing various foods and water microbiological analysis for many years, receiving the samples from Hygiene Department of Federal Ministry of Health (MOH) and other health services, like governmental health bureaus and health centers.

4.2. Study Samples

A total of 240 retail meat and dairy products were collected and investigated for *L. monocytogenes* during the period of August-December 2006. Sample size was calculated using the formula:

$$n = \frac{(Ž\ \alpha/2)^2\ (P(1-p)}{Sd^2}$$

By taking 5.1% *L. monocytogenes* prevalence rate in Addis Ababa, Ethiopia. (Molla *et al*., 2004)

Where P is the prevalence of *L. monocytogenes* in susceptible food sample

Sd is standard deviation of the observation which is allowable with Z α/2 (1.96), 95% confidence interval. (C I)

n = is the minimum sample size

$$n = \frac{(1.96)^2(0.51)(0.49)}{(0.05)} = 74$$

However, by taking the highest prevalent rate of *L. monocytogenes* in ice-cream samples from previous study, which was 19.6%, a total of 242 samples were calculated for this research and 240 samples were analyzed by approximately taking the nearest whole number.

4.3. Sample Collection

A total of 240 (60 from each of four types) food samples consisting of raw meat, raw milk, cottage cheese and cream cake samples of only Ethiopian products were purchased randomly from retail markets, shops, supermarkets and food vendors, of Addis Ababa and its surrounding towns, which are located, within the range of ≤140 kms, from Addis Ababa. The towns were Ambo (Hagere Hiywet), Nazret, Woliso, Fiche and Butajira whereas the sub cites are Addis Ketema, Kality, Kirkos, Lafto, Arada, Gulele and Yeka. The samples were purchased only from the sites where they were sold to the consumers. Fig. 3 and 4 shows the geographical location of these towns and sub-cities, from where the samples were purchased.

About5x (~100gm or ml) or 500 gm or ml from a lot, of each food type with a usual consumer size of the product was purchased and taken as a sample unit (Kiiyukia, 2003). All the food samples were purchased by the principal investigator and kept in ice box during transportation time. Then the samples were processed in the laboratory immediately upon arrival. However, when there was a delay, it was stored at freezing temperature, which then was thawed to room temperature, before processing. All information about the sample type, purchasing date/time and, place/site analyzing date/time, were registered (Annex I)


Yoboki
DJIBOUTI
Dikhil
Ali
Āysha
Desē
DENAKIL
Blue Nile
Debre Mark'os
GREAT RIFT VALLEY
Fichē
Debre Birhan
Dirē Dawa
Hārer
SHEWA
Addis Ababa
Debre Zeyit
ETHIOPIA
Nazrēt
ETHIOPIAN
PLATEAU
Āsela
Omo
Shabeelle
Jīma
Tarch'a Sodo
Āwasa
Goba
Gīnīr
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GULELE
YEKA
ADDIS KETEMA
ARADA
KOLFE KERANYO
LIDETA
BOLE
NEFAS-SILK LAFTO
AKAKI-KALITY

4. Laboratory culture analysis

4.4.1 Specimen Preparation

Each sample unit was agitated thoroughly to ensure the homogeneity of its contents and about 50 gm analytical unit (composite) was obtained aseptically by taking a portion (about 10 gm) from each 5 units into sterile petri dish for (cream cake) (only the cream parts of the cake were taken); by obtaining a portion (50 gm) from several location of the 500 gm sample unit using sterile spoon into sterile petri dish for (cottage cheese); by cutting and transferring a portion (50 gm) from several location of the 500 gm sample unit using sterile knife into sterile Petri dish (raw meat) and by agitating and directly drawing 25 ml from the 500ml sample unit into erlenymer flask (raw milk). Then, the composite samples were thoroughly mixed in the following manner; the raw milk was agitated; samples from raw meat were chopped using sterile knife; samples from the cream cake, were mixed using sterile scalpel and cheese samples were homogenized thoroughly using sterile scalpel.

4.4.2. Inoculation

Twenty five grams or milliliters of each sample from the composite mixture were weighed and transferred into 225 ml of previously prepared listeria enrichment broth (LEB) followed by mixing the suspended sample for 30 minutes, using a shaker at 2500 rev/min. (Kiiyukia, 2003). Finally the sample was kept inside incubator (TH 30 Incubator Shaker) and incubated at 30^0C for 48 hrs.

After 48 hrs of incubation, the inoculated LEB suspension was mixed by swirling or vortexing the Erlenmeyer flask and 0.1 ml was drawn and inoculated into 10 ml of Modified Fraser broth (MFB) and then incubated for 24hrs at 35 ^{0}C.

4.4.3 Isolation

As a continuation of the procedure from the selective enrichment broth, a positive MFB media with black /dark brown or dark green color was inoculated by taking a loop-full of the suspension into selective media Oxford agar (OXA), and PALCAM (Polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin Mannitol) agar and the plates were incubated at 35^0C for 24-48 hrs. Growth of 1-2 mm diameter black or black green colony with a black halo and black sunken center was taken as positive for *Listeria* species.

The colonies of *Listeria* were identified as per Falana (Falana *et al.*, 2003). On PALCAM agar typical colonies were grey-green with a black sunken center and a black halo. While on Oxford agar colonies appeared brown black or greenish black with a depressed center and a surrounding black halo.

When the colonies were grown and well isolated on the selective media, about 3-5 colonies from each medium were picked and suspended into TSY. It was incubated at 37^0C for about 1 hour till slight turbidity was visible and the suspension was inoculated into Tryptose yeast extract agar plates, to obtain pure culture and incubated for another 24hrs at 37^0C. Any grey-whitish colony was suspected to be positive for *Listeria.*

4.4.4 Biochemical tests

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

4.4.5 Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed for *L. monocytogenes* and other *Listeria* isolates by using Muller Hinton Agar. The common conventional antimicrobial drugs (amoxyclav, Co-trimexazole, cephalotin, chloramphenicol, cloxacillin, erythromycin, kanamycin, pencillin, rifampicin, steptomycin, tetracycline, vancomycin, gentamicin, and clindamycin) were tested. The method applied for antimicrobial testing was agar plate antibiotic disk diffusion method, using Kirby Bauer technique (MacGowan *et a*l, 1990; Antunes *et a*l.2002; Hansen *et a*l., 2005). About 2-3 pure colonies of the isolates were taken from the tryptone yeast extract agar and suspended in Muller Hinton broth and then, incubated at 37^0C for 1-2hrs. The suspension was then checked for the development of slight turbidity, against 0.5 MacFarland solutions (which is prepared by mixing 1% $BaCl_2$ and 1% H_2SO4, with a different proportion, and by taking the concentration, which is estimated to contain equivalent to about, 10^4 to10^6 organisms/ml). It was inoculated, by dipping a sterile cotton swab into it, and wiping on the Muller Hinton agar, according to the standard procedure (NCCLS) and then the antimicrobial discs were firmly placed on it and the plates were incubated at 37 ^{0}C for 24 hrs.

After the final incubation time, the zone of inhibition around each disc was measured, with the help of a caliper and the results were interpretated as sensitive, intermediate and resistant using a standard zone interpretative chart (NCCLS, 2000). Annex III

4.4.6 Quality control

L. monocytogenes (ATCC 35152), (CDC 91116) and (ATCC 19116) reference strains were used as positive controls for checking the efficiency of both culture media and antimicrobial susceptibility testing methods.

4.5. Statistical analysis

SPSS version 12.00 was used for data entry and statistical analysis. Since the data is a descriptive type, only frequency distribution and cross tabulations were used.

4.6. Ethical considerations

This research project was approved by the department of Microbiology, Immunology and Parasitology, Faculty of Medicine of AAU and FRPC.

4. 7. Application of the results

If *Listeria s*pecies are isolated from food samples, the result will be important evidence to start *Listeria* species identification, as a routine test within food microbiology laboratory of EHNRI (with in the country).

The antimicrobial profile of *L. monocytogenes* will be suggested for treating patients with *L. monocytogenes* related diseases, with potential antimicrobial drugs and those drugs with no action of treating(resistant) will be suggested to not be given or to be checked prior to prescribing the drugs.

It will be taken as baseline information, for cases of meningitis due to *L. monocytogenes*.

CHAPTER 5: RESULTS

In this cross-sectional study, a total of 240 food samples were purchased from randomly chosen 7 sub-cities of Addis Ababa and other 5 small surrounding towns, which are located within less than 140km distance from Addis Ababa. Details of the areas from where samples were purchased are given in table 4.

Table 4. The type and number of food samples purchased from 7 sub-cities of Addis and other surrounding towns.

Site of collection	Type of food sample				Total
	Cottage cheese	Cream cake	Raw milk	Raw meat	
Addis Ketema	5	5	5	5	20
Ambo town	5	5	5	5	20
Fiche town	5	5	5	5	20
Kality sub city	5	5	5	5	20
Kirkos sub city	5	5	5	5	20
Lafto sub-city	5	5	5	5	20
Nazret town	5	5	5	5	20
Woliso town	5	5	5	5	20
Arada	5	5	5	5	20
Butajira town	5	5	5	5	20
Gulele	5	5	5	5	20
Yeka	5	5	5	5	20
Total	60	60	60	60	240

From the total of 240 food samples collected, 66 (27.4%) *Listeria* species were isolated and among these 13 (5.4%) species were found to be *L. monocytogenes*. The number of *L. monocytogenes* and other *Listeria* isolated from different food samples is given in table 5. The percentages of listerial isolates made are shown in Fig.5.

L. monocytogenes was isolated from 11 raw meat samples (18.3%), and from 2 (3%) cake samples. On the other hand, of the total 66 listerial isolates including *L. monocytogenes*, 41(68.3%) were from the total 60 raw meat samples and 13 (21.6%) from cream cake samples, 6 each (10%) were isolated from both raw milk and cottage cheese.

Table 5: The number of *Listeria* species which were isolated from each food category

Type of species	Type of food sample				Total
	Cottage cheese	cream cake	Raw milk	Raw meat	
no growth	54	47	54	19	174
L. monocytogenes	0	2	0	11	13
L. ivanovii	0	1	0	0	1
L. innocua	3	5	3	15	26
L. seeligeri	0	1	1	1	3
L. welshimeri	0	2	0	6	8
L. grayi	0	1	0	0	1
L. murrayi	2	0	1	6	9
difficult to specify	1	1	1	2	5
Total *Listeria* isolates	6	13	6	41	66

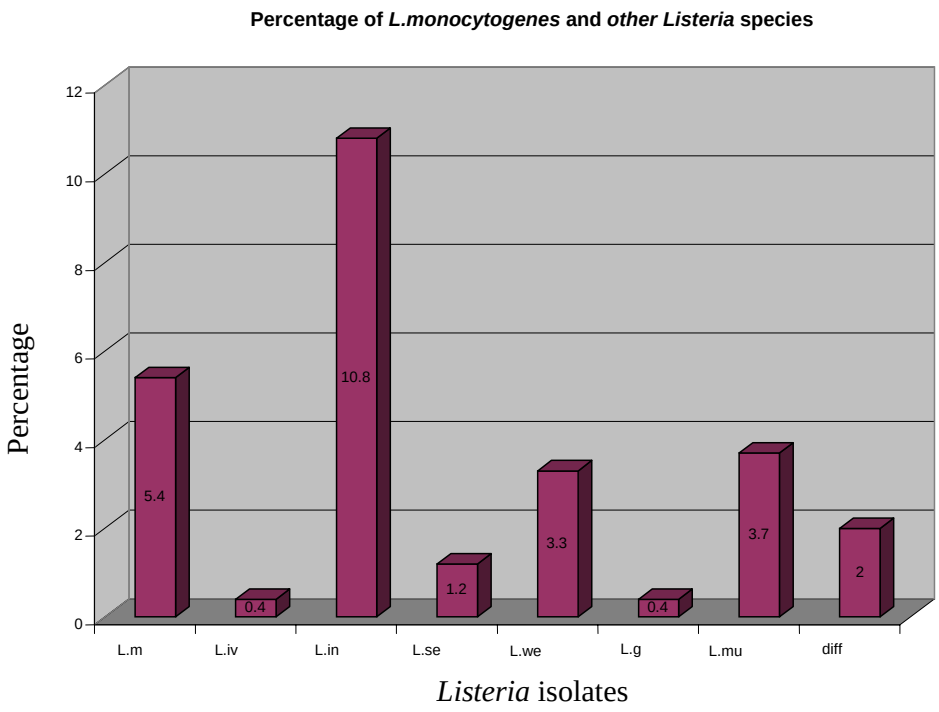

Figure 5: Percentage of *L. monocytogenes* and other *Listeria s*pecies isolated from the food samples

Key*; L. m: L. monocytogenes, L. iv: L. ivanovi, L. in: L. innocua, L. se: L. seelegeri, L. we: L. weleshemeri, L.g: L. grayi, L. mu: L. murrayi, diff: difficult to specify*

The isolation of *Listeria s*pecies was made for different food items collected from various sites. Table 6, shows the prevalence of these bacteria in different areas.

Table 6. The distribution of *Listeria* species isolated from 12 sites of collection

* indicates towns/sub cities where *L. monocytogenes* were isolated.

L. monocytogenes has been isolated from Addis Ketema, Kirkos, Lafto, Arada and Gulele sub-citys and Fitche town.

All *Listeria* isolates including *L.monocytogenes* were tested for their susceptibility to various antibiotics. The results of AST are given in table 7 and 8.

Table 7. Antimicrobial Susceptibility Test of listerial isolates

	No of isolates (in each spp.), which showed S, I, R against each antimicrobials													
L.spp	Ac	Clt	C	Cx	Co	E	K	P	R	S	T	Va	Ge	Cl
L.m) S I R	13 0 0	12 0 1	1 5 7	10 3 0	12 1 0	11 2 0	11 1 1	3 7 3	3 8 2	10 2 1	3 6 4	0 13 0	11 2 0	0 0 13
L.iv S I R	1 0 0	1 0 0	0 1 0	1 0 0	1 0 0	1 0 0	1 0 0	0 1 0	0 1 0	1 0 0	0 1 0	1 0 0	1 0 0	0 1 0
L.in S I R	25 1 0	18 3 5	2 16 8	19 7 0	25 1 0	19 5 2	18 7 1	7 9 10	4 15 7	19 5 2	8 14 4	24 0 2	24 2 0	0 3 23
L.s S I R	2 1 0	3 0 0	0 3 0	3 0 0	2 1 0	3 0 0	3 0 0	0 1 2	2 1 0	3 0 0	1 2 0	3 0 0	3 0 0	3 0 0
L.w S I R	0 8 0	5 1 2	0 5 3	6 2 0	7 1 0	5 3 0	5 3 0	2 1 5	0 4 4	5 3 0	2 5 1	8 0 0	6 2 0	0 1 7
L.mu S I R	8 0 1	3 4 2	0 3 6	4 5 0	8 1 0	4 5 0	3 5 1	3 1 5	0 3 6	3 5 1	0 6 3	9 0 9	5 2 2	0 0 9
L.g S I R	1 0 0	0 1 0	0 0 1	0 1 0	0 1 0	0 1 0	0 1 0	0 0 1	0 0 1	0 1 0	0 1 0	1 0 0	1 0 0	0 0 1

Key: L.m: Listeria monocytogenes, L.iv: Listeria ivanovi, L.in: Listeria innocua, L. s: Listeria seelegeri: L. w: L isteria weleshemeri, L.g: L.grayi, L.mu: L.murrayi, (n): no of isolates

S: Sensitive, I: Intermediate, R: Resistance, spp: species, abials: antimicrobials

Ac: Amoxyclav, Clt: Cephalothin, C: Chloroamphenicol, C: Cloxacilin, Co: Co-trimaxazole, E: Erythromycin, K: Kanamycin, P:Pencillin, R: Rifampcin, S: Streptomycin, T: Tetracycline, Gen: Gentamicin, Va: Vancomycin, Cl: Clindamycin

Table 8: Percentage of susceptibility of *L. monocytogenes* isolates to different antibiotics tested.

Antimicrobial agent	No/(%)of susceptible strains
Amoxyclav	13 (100%)*
Cephalothin	12 (92.3%)*
Chloramphenicol	6 (46.1%) **
Cloaxcillin	13 (100%)*
Co-trimaxazole	13 (100%)*
Erythromycin	13 (100%)*
Kanamycin	12 (92.3%)*
Pencillin	10 (76.9%) **
Rifampicin	11 (84.6%)
Streptomycin	12(92.3%)*
Tetracycline	9 (69.2%) **
Vancomycin	13 (100%)*
Gentamicin	13 (100%)*
Clindamycin	0**

Key; *: drugs which have shown ≥ 90% sensitivity

** “” ≥20% resistance

CHAPTER 9: DISCUSSION

Listeria monocytogenes continues to be of worldwide interest to the food industry and regulating agencies, to the scientists in various disciplines, and to the consumers of ready to eat foods. Sporadic cases of listeriosis continue to occur worldwide and there have been several outbreaks of the disease associated with food in USA and Europe. The findings of the present study showed that *L. monocytogenes* and other species of this genus are quite prevalent in different types of food in Ethiopia. In developing countries of Africa and Asia, much attention is being paid to food associated pathogens other than *Listeria* because they cause high morbidity and mortality. But the results of the present study and that of Molla *et al*., 2004 showed that *Listeria* in food industry existed all the time in these regions like that of USA and Europe but anyone hardly looked for it.

Heightened public health concern regarding the presence of *L. monocytogenes* in food prompted the United States Food and Drug Administration to initiate a series of *Listeia* surveillance programs. Subsequently the discovery of this pathogen in many varieties of domestic and imported cheese, in ice cream, and in other dairy products prompted the numerous product recalls, which in turn have lead to staggering financial losses for the industry. These listeriosis outbreaks together with a subsequent epidemic in Switzerland involving consumption of soft ripened cheese and discovery of *L. monocytogenes* in raw and ready- to-eat meat, poultry, sea food, and vegetables have underscored the need for additional information concerning food borne listeriosis (Ryster and Marth, 1999). Similar conditions may be prevailing in developing countries so the present study was

undertaken to initiate interest of medical persons and scientists involved in food hygiene or food microbiology to the problem of listeriosis.

For this study, the following four food samples were chosen and tested, for the presence of *Listeria* because these food types are widely consumed throughout the country, while they are raw or as ready to eat food (processed food), especially in relation to Ethiopian traditional culture. For example, most Ethiopians consume raw meat and ready to eat cheese on holidays, and religious festivals and social occasions, like wedding etc. The same is true for raw milk; mostly it is used while it is uncooked, especially in rural regions, which is very dangerous and well known to harbor millions of different microorganisms and parasites. Similarly, cream cake is also consumed after baking and decorating with different colored ready to eat creams (milk product), which is again exposed for bacterial contamination through poor sanitary conditions and is largely consumed by children.

The other reason why these samples were chosen was because all the four food samples are cattle products. *Listeria* is mostly associated with cattle and has an impact on animal health. Therefore, it is an important pathogen in veterinary practice. It causes abortion and meningitis in cattle and sheep and variety of diseases in other mammals. Milk and dairy products were the first and are among the most extensively studied foods in relation to food-borne listeriosis (Rocourt and Cossart, 1997). The same is true for raw meat. As it is already known, *L. monocytogenes* was first detected from animals, before it was isolated from food samples or humans or other living things.

The predominant bacterium isolated in the present study was *L. innocua,* 26(10.8%), which is the most common isolate in this group reported by several workers (Molla *et al.*, 2004; Opinions, 1999). It was known to be the highest prevalent *Listeria* species reported in several researches (Safdar & Armstrong, 2003). Similarly in this study it was isolated

from 26 food samples, followed by 13 *L. monocytogenes* species, which ranked in the second position.

In Ethiopia, the first study was undertaken by Molla *et al*., (2004) and reported, several species of *Listeria* from different food samples as 32.6% and all the known *Listeria* species were isolated from the examined food samples. In the similar manner, the present study also confirms the presence of *L. monocytogenes* and other species of this genus in many ready to eat foods and points to the danger of our habit of eating raw food. *L. monocytogenes* was isolated in 5.4% in our study and 5.1% in earlier work. This result is comparable with results of surveys undertaken in other countries. In China, *L. monocytogenes* was isolated in 5.79%, from food products of China (Chao, *et al*., 2007).

On the other hand, those isolates, which grew and showed similar growth characteristics on the selective media, like other *Listeria* species, but did not coincide with the actual biochemical characteristics at the species level, were termed under the name of 'difficult to specify'. The reason here is, it is difficult to exclude these isolates from the report, because their culture morphological characteristics looked like the *Listeria* species, but they failed to meet any of the known species category by biochemical tests. This might be the isolates exhibit weak reactions for most of the biochemical tests and this could make detection and categorization difficult. If the facilities were available and there was a chance of using other methods like sero-typing or PCR techniques; it might have been possible to categorize these isolates as *Listeria* species. It would have been possible to identify these isolates by other criteria like antigenic classification or serological characterizations with specific antigens, but unfortunately these facilities were not available so we have described these *Listeria* isolates as unclassified.

The distribution of *Listeria* species in each food category is quantified in the following manner. About 60 cottage cheese samples were tested and only two types of *Listeria* species with total no of 5 (8.3%) isolates were found. These are; *L. innocua* 3(5%), *L. murrayi* 1(1.6%) and one unidentified *Listeria* species was isolated, but none of the

pathogenic *L. monocytogenes* was isolated from these samples. This might be due to the freshness of these cheese types.

As reported in several surveys, soft ripened cheese appears to be the most suitable both to contamination and growth of *L. monocytogenes* than the cottage cheese (WHO, 1988). Similarly, most surveys suggest that 1 to 10% of cheeses (especially soft cheeses) are contaminated with *L. monocytogenes* (Lovett *et al.*, 1987). So like earlier observations, we also concluded that the contamination level of cottage cheese is low as compared to other types of ripened cheese because cottage cheesees have acidic pH which does not support the growth of bacteria (Farber *et al*., 1987).

Similarly, from the 60 cream cake samples tested; 13 (21.5%) were found to have six types of *Listeria* species, these were *L. ivanovii* 1(1.6%), *L. grayi* 1(1.6%), *L. seeligeri* 1 (1.6%), *L. monocytogenes* 2(3.3%), *L. welshimeri* 2(3.3%), and *L. innocua* 5 (8.3%). This indicated how often these food types are over exposed to different types of pathogenic bacteria including *L. monocytogenes*. This finding is in agreement with other surveys involving ice cream and cream cake samples, which are commonly stored in refrigerators (Molla *et al*., 2004). They are frequently found contaminated with *L. monocytogenes* with 0.3 to 2% of samples tested (Rocourt and Cossart, 1997). It is well known that low refrigerator temperature supports the growth of *L. monocytogenes.* In another survey, its prevalence was indicated from zero to approximately 5.5% of products tested (WHO, 1988). It may be of interest that refrigeration of clinical samples is the common method of enrichment of *L. monocytogenes* from animal tissues before isolation. In most cases, cream cake contamination has been attributed to post process contamination and also the components of cake (egg, milk, butter), which contain high nutritional value ingredients, can serve as good nutritional and enriched medium for growth of bacteria and play a major role, by providing support for bacterial multiplication.

However, this finding showed a lower percentage when compared to the previous Ethiopian work (Molla *et al*., 2004). They have reported that, *Listeria* spp were isolated as 43.5% and *L. monocytogenes* 19.6% from 40 ice cream samples tested, which showed

a higher prevalence of *Listeria* in ice creams. This discrepancy from present work might be justified as type of samples was different; ice cream versus cream cake probably. As we know, ice cream has relatively higher cream content than cream cake samples.

In the case of raw milk, *Listeria* species were isolated with the total number of 5(8.3%); these are *L. seeligeri* 1(1.6%), and *L. murrayi* 1 (1.6%) *L. innocua* 3 (5%), However, no *L. monocytogenes* species was isolated from this source like cottage cheese. Most literatures reported the prevalence of *L. monocytogenes* as 0-2.2 % (opinions 1999), greater than 2 % (Rocurrt *and Cossart,* 1997), (0-10%) (Todar's, 2003). The present study is in agreement with these reports. One of the reasons for low prevalence of *Listeria* species in raw milk may be the early consumption of raw milk after it is obtained from animal. Therefore, there are few chances of its contamination from handlers or environment.

In the present study, raw meat was found to be one of the richest sources of *Listeria* organisms, including *L. monocytogenes.* Out of the 60 raw meat samples tested, almost more than half of the samples were contaminated with *Listeria* species in which 11/13 *L. monocytogenes* were isolated and all *Listeria* species except *L.ivanovii* and *L.grayi* were identified. This shows how the meat samples are exposed to poor sanitary conditions, during slaughtering, selling or meal preparation. Also most of our cattle products are contaminated by *Listeria* species (Molla, *et al*., 2004) which shows high prevalence of *L. monocytogenes* in combined with Ethiopian practice of eating raw meat and one can easily imagine how eating raw meat is dangerous to humans. Similarly, this finding is in agreement with other studies as Rocourt & *Cossart,* 1997, who have reported isolation of *Listeria* species from raw meat as high as 50% and Opinion (1999) in 22%. It was noted that raw cured meat products were significantly contaminated with *L. monocytogenes* than cooked meat products, 13.7% and 4.9%, respectively (Uyttendael *et al*., 1997).

As indicated above, none of the *L. monocytogenes* species were isolated from both raw milk and cottage cheese and also a very small number of other *Listeria* species were isolated, from these samples. Moreover these samples were collected from those regions,

where the raw meat and cream cake samples were collected; (where *L .monocytogenes* were also isolated), however, the samples were contaminated but with minimum number of the organisms. The explanation here is, raw milk contains natural bacterial inhibitors such as the lactoperoxidase system (LPS) and specific microflora. The microbiological composition of the raw milk, in terms of thermophilic lactobacillus and yeast might have the inhibitory effect on the growth of *L. monocytogenes* (Gay and Amghar, 2005)

The above 240 food samples were collected from randomly chosen different 7 sub-cities of Addis Ababa, the capital city of Ethiopia and its surrounding five towns. *L. monocytogenes* was isolated in 6 of the 12 regions. These are from Addis Ketema, Kirkos, Lafto, Arada and Yeka sub-cities and only from Fiche town. The highest number of isolates of *L. momocytogenes* was obtained both from Addis ketema and Kirkos sub-cities. Interestingly both had similar numbers of isolates which was three. Fiche town and Lafto regions rank second to the former places where two isolates were obtained in each. However, only one isolate was found from Arada sub-city. It is a bit difficult to explain this observation, because it is assumed that Addis Ababa sub-cities have a better hygiene and better health information than surrounding towns. The suggested explanation here is, it might be because of the freshness of sample from towns. Moreover, this study was time and resources bound, therefore much conclusion can not be made from this work except *L. monocytogens* is present in different types of food. If such study is made on very high numbers, more interesting observations may be obtained.

In general, *Listeria* species have been isolated from all 7 sub-cities of Addis Ababa and from four regions of other small towns' except Naz-ret. None of the *Listeria* species were isolated from the 20 food samples tested in this town. It is difficult to explain this finding in this study because the sample size was too small

The highest number of *Listeria* species was found from Butajira followed by Arada sub city. However, none of them was found to be *L. monocytogenes*. Based on this study it was shown that all types of *Listeria* species are prevalent in Addis Ababa and in other small towns as exemplified.

In the present study, the main objective was to find out the distribution of *L. monocytogenes* and it was proved to be prevalent in those indicated regions, with 5.4%. This prevalence is similar to the earlier work, (Molla *et al.*, 2004), where it was 5.1%. It could be safely said that these findings are almost similar in detection of *L. monocytogenes,* even though the investigated samples were somewhat different. The major difference, which was observed between the two studies, is the highest number of *Listeria* and *L. monocytogenes* species which were obtained from raw meat samples in this study, while ice cream samples revealed the highest number, reported in the earlier study. However, the results of the cottage cheese samples in both projects were observed to be similar, though number of isolates was much smaller when compared to other types of foods tested.

The antimicrobial profile of *L. monocytogenes* were also studied based on the standard Kirby Bauer method as indicated in the methodology section, and it was done for all *Listeria* isolates. However, there is no standard interpretation chart for all types of drugs studied, for checking the breakpoint (cut off) value of the results for *L. monocytogenes*, which is our target. Therefore, for most of the drugs; the results have been interpretated based on the standard breakpoints reported by NCCL's for Gram positive organisms, and only for penicillin, Chloramphenicol, and tetracycline, the actual zone of interpretation chart for *L. monocytogenes* was used (Rodas-Sua´rez *et al.*, 2006; Zhang *et al*., 2007).

Therefore, based on these background, all *L. monocytogenes* isolates were found to be sensitive and intermediate to most of the drugs tested; amoxyclav, Co-trimaxazole, cephalothin, cloxacillin, erythromycin, kanamycin, vancomycin and gentamicin. This finding is in agreement with most surveys undertaken on the sensitivity of *Listeria* to antimicrobial drugs (Safdar *and* Armstrong, 2003). In Denmark, there was a study which reported susceptibility (sensitivity) of *L. monocytogenes*, to penicillin G, ampicillin, meropenem, gentamicin, sulphamethoxazole, trimethoprim, erythromycin, vancomycin, linezolid, chloramphenicol and tetracycline except ciprofloxacin (Troxiler *et al.*, 2000; Hansen *et al.*, 2005).

However, it was found that all of 13 isolates were resistant to clindamycin, and also to certain extent to choroamphenicol, pencillin G and tetracycline in the similar manner with other reports (Safdar & Armstrong, 2003; Antunes *et al*., 2002). Tetracycline resistance of *L. monocytogenes* has also been reported with 8.4% (Li. Q *et al.*, 2005; Zhang *et al.*, 2007), in addition to its resistance to sulfonamide (73%) and ciprofloxacin (1.8%). Multi resistant strains towards ampicillin, erythromycin, tetracycline, dicloxacillin, and trimethoprim-sulfamethoxazole have also reported (Rodas-Sua´rez *et al.*, 2006).

In some findings, cephalothin is indicated as a drug to which *L. monocytogenes* is resistant (Zhang *et al*., 2007), but in this study it was shown to be resistant only to a single isolate.

Similarly, the antimicrobial profile of other *Listeria* species was studied and observed that, there was not much difference between the human pathogenic *L. monocytogenes* and other non pathogenic *Listeria* in their antimicrobial profile. *L. innocua* which is considered to be non-pathogenic organism, but it was observed that it showed similar resistance to most of the drugs tested, like *L. monocytogenes*. This organism was observed to be resistant to penicillin, tetracycline and also to Chloramphenicol. Most of the non pathogenic species of *Listeria* like, *L. seeligeri and L. welishimeri* isolated from food samples showed resistance to commonly prescribed antibiotics like tetracycline, etc. They showed resistance to one or two of the drugs tested. This indicated how these organisms are widely distributed in the society and infect many people including different foods and also the environment. They have adapted to most of the drugs, which are largely consumed by people through out the society, following blind antimicrobial treatment.

In addition to this, it is generally known that, all *Listeria* species were naturally resistant to certain drugs individually or as a group. For example, *L. grayi* is naturally resistant to trimethoprim and co-timoxazole; least susceptible to rifampicin and most susceptible to

quinolones, whereas *L. ivanovii* is naturally resistant to most quinolones and naturally sensitive to fosfomycin, whereas *L. innocua* and *L. monocytogenes* are naturally resistant to fosomycin (Troxler et al., 2000).

In medical practice too, clinical cures in listeriosis have been obtained with ampiciliin, erythromycin or with trimethoprim-sulfamethoxazole. Ampicillin plus gentamicin is often recommended for therapy (Brooks *et al*., 2004).

CONCLUSIONS AND RECOMMENDATIONS

In general, the main objective of this thesis was to look for the presence of *L. monocytogenes* in ready to eat foods, in Ethiopian conditions. *L. monocytogenes* and other *Listeria* species were isolated in these examined food samples almost with similar percentage like the developed countries. From this understanding and the peculiar characteristics of listeriosis (high case fatality rate, emerging, opportunistic, sporadic and causes death in immuno-compromised individuals); we can draw the conclusion that it is present in Addis Ababa and also in some surrounding towns, it can pose a threat to human life and can cause similar outbreaks of morbidity and mortality here too. Who knows many people who have been suffering from unknown causes of meningitis, abortion, still birth, septicemia, endocardities, and so on, might be infected by *L. monocytogenes*. Therefore, creating public awareness by disseminating the information is necessary. In conclusion, serious precautions regarding the food type, storage system and

proper cooking should be considered while handling of food, in order to control listerial food contamination.

In addition, as explained above *L. monocytogenes* is proved to be sensitive to many and available drugs. So examining food samples especially of raw- ready to eat food, must be made as routine way in food microbiology laboratories. In hospitals, suspected cases of meningitis both in adults and neonates must be investigated for *L. monocytogenes* so that proper treatment can be undertaken as well as the prevalence of this disease may be known in Ethiopia.

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

- An extensive survey on the prevalence of *Listeria* species in ready to eat foods in whole of Ethiopia must be undertaken.

- All food samples (Raw meat, cream cake, raw milk and cheese) must be investigated as a routine test for *L. monocytogenes* in food microbiology or qualified laboratories
- All suspected patients for meningitis, Abortion, septicemia…,should be investigated for listeriosis
- As far as possible consuming foods directly from refrigerator unless treating them with sufficient heat (proper cooking) and should be avoided.
- Antimicrobial susceptibility test must be done for all *L. monocytogenes* isolates, whether they are identified from food samples/ environment or from clinical samples.
- Attention has to be given to the habit of consuming raw meat, raw milk, etc at nation wide.

Limitations of the study

- The present study had financial and time constraints so it could not include large areas or numbers of food samples
- Only available antimicrobial agents were used for susceptibility testing.
- Serological classification of listerial isolates could not be done because of lack of finance.

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10. Appendix I

Every sample was purchased by the principal investigator and any information about the sample and result report was filled in this format.

Date__/__/__

1. Sample Identification

Type of sample _____

Purchasing date/time _____/_____

Site; Town_____Kifle Ketema _____Kebele_____

Arrival time/date (Lab.) _____/_____

Processing date/time ` _____

2. Culture result

Listeria Enrichment broth _____detection time _____date_____

Modified Fraser broth _____detection time _____date_____

PALCAM agar _____detection time _____date_____

Oxford agar ~~detection time date~~ ____ _____

3. Biochemical test date/time_____

Haemolysis _____

CAMP test _____

L-Rhamnose _____

D-Xylose _____

Mannitol _____

4. Result

L.monocytogenes Positive_____Negative_____

L.innocua Positive _____Negative_____

L.ivanovii Positive _____Negative_____

L.welshemeri Positive ~~Negative~~ _____

L.seelegeri Positive ~~Negative~~ _____

L.murayi Positive ~~Negative~~ _____

L.grayi Positive ~~Negative~~ _____

5. Antimicrobial susceptibility test result

Types of drug	Sensitive(_mm)	Resistant(_mm)	Intermediate(__mm)
Amoxyclav			
Cephalothin			
Co-trimexazole			
Chloramphenicol			
Cloxacillin			
Erythromycin			
Gentamicin			
Kanamycin			
Rifampcin			
Streptomycin			
Tetracycline			
Clindamycin			
PenicillinG			
Vancomycin			

Comment_____

_____

_____

Date ____/_____/_____

Signature_____

Appendix II

Principles and Procedures of the Media and Broths used

1. Pre-enrichment

Listeria enrichment broth was used (LEB)

Specifications; KM 10505

Use; An enrichment broth for detection of *Listeria* spp.

Ingredients;

Peptone mixture	20 gm
Yeast extracts	6.0 gm
Sodium chloride	5.0 gm
Potassium dihydrogen phosphate	2.5 gm
Glucose	2.5 gm
Nalidixic Acid	0.04 gm
Acriflavin HCL	0.015
Cyclohexamide	0.05

pH; 7.3+/- 0.2

Preparation;

36.1 gm of powder was weighed and added to 1 lt of deionized water (conductivity <10ms) and then warmed until complete dissolution. It was mixed well and 225 ml was distributed into each of 250ml erlenymer flasks and sterilized by autoclaving at 121 ^{0}C for 15'.

Selective enrichment

Listeria Fraser broth

Specification KM 10335

Use; for isolation and enumeration of *Listeria* species

Ingredients;

Peptone mixture	10 g
Yeast extract	5 g
Sodium chloride	20 g
KH2PO	1.35 g
Na2HPO	9.5 g
Beef extract	5.0 g
Nalidixic Acid	0.010 g
Acriflavin Hcl	0.0125 g
Aesculine	1.0 g
Lithium chloride	3.0 g

PH= 7.2+/_ 0.2

Preparation;

27.4 gm of the powder was weighed and added to 500ml of the deionized water (conductivity<10ms), then it was well mixed and sterilized by autoclaving at 121^0C for 15'. After sterilization, it was allowed to cool to around 47^0C and previously prepared and filtered (sterilized), 5 ml of 5% Ferric ammonium citrate* supplement was added to this broth. Then it was well mixed and 10ml of the broth was aseptically dispensed into sterile tubes.

*Ferric ammonium citrate (17% Fe)-MERCK

3. *Listeria* isolation agar; two selective media were used for this purpose ;

3a. Oxford Agar;

Specification: KM1049

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

Ingredients;

Coloumbia agar	42.0 g
Aesculine	1.0 g

Ferric Ammonium citrate 0.5 g

Lithium chloride 15.0 g

pH= 7.2 +/- 0.2

Preparation

5 g of the powder was weighed and added to 1 lt of deionized water and then it was allowed to soak for 10' and swirled to mix and sterilized by autoclaving at 121 ^{0}C for 15'. It was kept at room temperature until it cooled to around 47^{0}C and 2 vials of dry powder of the selective supplement KM 'SO49 was suspended with 1 ml distilled water and added into this medium. At last this prepared medium was thoroughly mixed by agitating and it was pour plated into sterile petridish.

KM SO49 supplement contains;

CCNAF selective supplement (MICRO TRADE)

Formula; CEFOTITAN 1 mg

COLISTIN 10 mg

FOSOMYCIN 5 mg

ACRIFLAVINE 2.5 mg

NATAMYCIN 12.5 mg

3b. **PALCAM (Polymixin acriflavin lithium chloride ceftazidime, aesculin and mannitol) agar base**

Specification; KM S079

Use; An important selective differential medium for the isolation of *L.monocytogenes* from food, clinical and environmental specimens

Ingredients:

Columbia peptone mix 23.0 g

Aesculine 0.8 g

Ferric ammonium citrate 0.5 g

Lithium chloride 15.0 g

Corn starch 1.0 g

Yeast extract 3.0 g

Mannitol	10.0 g
Sodium chloride	5.0 g
Glucose	0.5 g
Phenol red	0.08 g
Agar	12.0 g
	pH=7.2 +/- 0.2

Preparation

1 liter of PALCAM medium was prepared by weighing and adding of 70.8 g of the powder into one liter of deionized water, followed by mixing and sterilizing of the medium at 121^0C for 15'.Then, it was allowed to cool to around 47 ^{0}C and 2 vials of the selective supplement KMSO79 was added (as in OXA case), mixed and pour plated. At this level the PALCAM medium was used after keeping for some time to allow drying of the medium.

KMSO79 selective supplement- PAC (MICRO TRADE)

Formula;	Polymixin B	6.25 mg
	Cetrazidine	10 mg
	Acriflavine	2.5 mg

4a. Tryptose yeast extract agar

Specification; KM 1116

Use: an agar for performing total viable count by the pour plate method

Ingredients;

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
	pH 7.0+/-0.2

Preparation; 23.5 g of the ingredients was suspended in1 lt of the deionized water and boiled with frequent stirring. Then, it was dispensed into screw-capped tubes and

autoclaved at 121^0C for 15 minutes and the rack was kept in slant position in order to prepare slants.

Appendix III. Zone Diameter Interpretive Standards (Kirby Bauer disk diffusion method NCCLS, 2000)

Antimicrobial agent	Symbol	Disc content (µg)	Zone Diameter (mm)			
			S	I	R	Comments
Amoxyclav (Amoxicillin-Clavulanic acid)	AC	30(20/10)	≥ 18	14-17	≤ 13	G.P
Cephalothin	Ch	30	≥18	15-17	≤14	G.P
Chloramphenicol	C	30	≥21 ≥ 18	18-20 13-17	≤ 18 ≤ 12	L.m * G.P
Cloxacillin	Co	5	≥ 14	10-13	≤ 9	G.P
Co-trimexazole (Trimethoprim-sulfamethoxazole)	SXT	25 (1.25/23.75)	≥ 16	11-15	≤ 10	G.P

Erythromycin	E	15	≥ 23 ≥23 ≥ 18	20-22 14-22 14-17	≤ 20 ≤ 13 ≤13	L.m* G.P. GP
Kanamycin	K	30	≥ 18	14-17	≤ 13	G.P
Pencillin G	P	10 units	≥34	31-33	≤ 31	L.m*
Rifampicin	R	5	≥ 20	17-19	≤ 16	G.P
Streptomycin	S	10	≥ 15	12-14	≤ 11	G.P
Tetracycline	T	30	≥ 25 ≥19	22-24 15-18	≤ 22 ≤ 14	L.m* G.P.
Vancomycin	Va	30	≥ 12	10-11	≤ 9	G.P.
Gentamicin	G	10	≥ 15	13-14	≤ 12	G.P.
Clindamycin	Cd	2	≥ 18	14-17	≤ 13	G.P.

Keys; G.P- Gram positive

L.m- Listeria monocytogenes

S = Sensitive I = Intermediate

R = Resistance

Appendix IV DECLARATION

I declare that this MSc Thesis is my own original work and has not been presented for a degree, in any other university.

Investigator: Firehiwot Abera Derra

Signature: _____

Date of Submission _____

This thesis has been submitted for examination with our approval as University advisors:

1. Prof DP Monga (PhD)

Signature_____ Date _____

2. Dr Abera Geyid (MSc, PhD)

Signature_____ Date _____

3. Dr Abebe Mache (MSc, PhD)

Signature _____ Date _____

Place of submission: Department of Microbiology, Immunlogy and Parasitology
Faculty of Medicine, Addis Ababa University
Addis Ababa, Ethiopia
March, 2008

Fig. 3: Towns from where food samples were purchased: Fiche=North Shewa, about 135 km from AA; Ambo=West Shewa, about 125 km from AA; Nazret=100 km southeast of Addis Ababa; Butajira (Ara Shatan) about 140 km south of Addis Ababa and Woliso about 115 km south west of AA.

Table 6. The distribution of *Listeria* species isolated from different sites of collection

Site of collection	Type of species							
	L. monocytog-enes	*L. ivanovii*	*L. innocua*	*L. seeligeri*	*L. welshimeri*	*L. grayi*	*L. murrayi*	difficu speci
Addis Ketema*	3	0	0	0	0	0	2	0
Ambo town	0	0	3	0	0	0	0	2
Fiche town*	2	0	2	0	1	0	0	0
Kality sub city	0	0	3	0	0	0	3	0
Kirkos	3	0	2	0	0	0	0	1

s-city*								
Lafto sub-city*	2	1	2	0	2	0	0	0
Nazret town	0	0	0	0	0	0	0	0
Woliso town	0	0	1	0	1	0	0	0
Arada*	1	0	7	0	0	1	0	0
Butajira town	0	0	2	3	3	0	0	2
Gulele	0	0	2	0	0	0	3	0
Yeka*	2	0	2	0	1	0	1	0
Total	**13**	**1**	**26**	**3**	**8**	**1**	**9**	**5**

* indicates towns/sub cities where *L. monocytogenes* were isolated (Addis Ketema, Kirkos, Lafto, Arada and Gulele sub-cities and Fiche town).

Fig 4: Sub cities of Addis Ababa, from where food samples were collected
Adopted from Addis Ababa city of Administration Official website.
www.addisababacity.gov.et