PREVALENCE AND ANTIBIOGRAMS OF *LISTERIA MONOCYTOGENES* ISOLATES FROM RAW MILK AND YOGHURT IN ZARIA, KADUNA STATE, NIGERIA

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DECLARATION

I declare that the work presented in this thesis titled **"PREVALENCE AND ANTIBIOGRAMS OF *LISTERIA MONOCYTOGENES* ISOLATES FROM RAW MILK AND YOGHURT IN ZARIA, KADUNA STATE, NIGERIA"** has been carried out by me in the department of Veterinary Public Health and Preventive Medicine. The information derived from the literature has been duely acknowledged in the text and a list of refrences provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Veronica Chizoba GABRIEL

……..	…….....	……..
Name of Student	**Signature**	**Date**

CERTIFICATION

This thesis entitled **"PREVALENCE AND ANTIBIOGRAMS OF *LISTERIA MONOCYTOGENES* ISOLATES FROM RAW MILK AND YOGHURT IN ZARIA, KADUNE STATE, NIGERIA"** by Veronica Chizoba GABRIEL meets the regulations governing the award of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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Prof. J.K.P. Kwaga **Chairman, Supervisory Committee**	**Signature**	**Date**
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DEDICATION

To Divine, David and Delight.

My blood, my Love, my Children,

Who has made the writing of this thesis so much easier with their words of encouragement.

Lord, your word calls a mother to Love,

I have since 2006. I do right now. I forever will.

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Mercy, Grace and Love be multiplied to you in Jesus name.(Amen).

To CHIZOBAM (Jehovah, my Savior), it can NEVER be complete without You. I LOVE YOU LORD!

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ABBREVIATIONS

act A	Actin A gene
CDC	Centers for Disease Control and Prevention
HACCP	Hazard Analysis and Critical Control Point
*hyl*A	Haemolysin A gene
*inl*A	Internalin A gene
LAB	Lactic Acid Bacteria
LIPI 2	*Listeria* Pathogenicity Island.
LLO	Lysteriolysin O
MPA	Monocytosis Producing Agent
PBP3	Penicillin Binding Protein 3
PCR	Polymerase Chain Reaction
PI-PLC	Phosphatidylinositol-specific Phospholipase C
UHT	Ultra High Temperature

ABSTRACT

Listeria monocytogenes is a food borne pathogen frequently associated with the consumption of contaminated food. Severe and life threatening human diseases caused by *L. monocytogenes* has been reported throughout the world. The present study evaluated the prevalence and antibiograms of *Listeria monocytogenes* isolated from yoghurt and raw milk sample in Zaria, Kaduna State. One hundred and fifty eight samples each of yoghurt and raw milk making a total of three hundred and sixteen samples were purchased within Zaria, Kaduna State. The samples were bacteriologically analyzed in the laboratory for the presence of *L. monocytogenes* using conventional biochemical tests and the use of Microgen^{TM} Listeria -ID System to further confirm the isolates. The conventional biochemical tests identified 36 isolates of *Listeria* species out of which ten were *Listeria monocytogenes* from the raw milk samples, giving a prevalence of 3.2%. However, the Microgen^{TM} Listeria –ID System failed to identify 9 out of the 10 *Listeria monocytogenes* isolates. Antibiotic susceptibility profiles showed that all the isolates were resistant to three or more antibiotics except for the aminoglycoside (gentamicin) to which all the isolates were susceptible (100%). Nine different resistance patterns were observed with resistance to ampicillin being the most predominant (100%). The minimum Multiple Antibiotic Resistant (MAR) index was 0.27 indicating that isolates were from a source of high antibiotics abuse. The ages of the animals were not significant to the detection of *Listeria* spp ($p=0.346$) whereas the pH of the milk was significant to the detection of *Listeria* spp ($p=0.049$). The findings of this study suggest the possible role of raw milk in the transmission of antimicrobial resistant strains of *Listeria monocytogenes* to human.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Listeria species are Gram positive, ubiquitous bacteria widely distributed in the environment. The genus *Listeria* currently contains ten species: *Listeria* (*L*.) *monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi* (Boerilin, *et al*., 1992; Jones and Seeliger, 1986), *L. marthii* (Graves *et al*., 2010), *L. rocourtiae* (Leclercq *et al*., 2010), *L. Fleischman nii* (Bertsch *et al*., 2013), and *L. weihenstephanensis* (Lang Halter *et al*., 2013). Recently, five new *Listeria* species (*L. floridensis* sp. Nov., *L. aquatic* sp. Nov., *L. cornellensis* sp. Nov., *L. riparia* sp. Nov., and *L. grandensis* sp.) were identified from agricultural and natural environments in the United States (Henk *et al*., 2014). No haemolytic activity or PI-PLC activity was detected among the new species suggesting they lack the genes (*hly* A and *plc* A) associated with virulence in *Listeria monocytogenes* and *Listeria ivanovii*. The absence of virulence genes found in the *prf* A cluster (Schmid *et al*., 2005) or LIPI-2 (Dominquez- Bernal *et al*., 2006) from the draft genome, further support that none of the new species described are pathogenic.

Of these species only *L .monocytognes* is of significance in public health (Farber and Peterkin, 1991; Swaminathan, 2001).

L. monocytogenes is an important pathogen in human and veterinary medicine, causing abortion and encephalitis in sheep and cattle and a variety of diseases in other mammals, birds and fish (Kalorey *et al*., 2008). *L. monocytogenes* is the causative agent of listeriosis,

a serious foodborne disease primarily associated with the consumption of processed foods that require no further cooking by the consumer (McLauchlin, 1996; Low and Donachie, 1997; Schlech, 2000). Human acquisition of listeriosis from animal sources has been shown to occur as an occupational hazard especially in farmers, butchers, poultry workers and veterinary surgeons but most human infections are probably food-borne primarily associated with the consumption of contaminated milk and dairy products. Dairy products such as milk, cheese, ice cream and butter have all been implicated (Adetunji and Arigbede, 2011).

Symptoms of listeriosis in human include diarrhoea, fever, headache, and myalgia (mild symptoms). In cases of invasive listeriosis, meningitis and septicaemia are commonest forms of the disease which in pregnant women can lead to abortion and stillbirth (Aygun and Pehlivanlar, 2006). In general, pregnant women and their unborn children, newborns, immune compromised persons and the elderly are at great risk for listeriosis, which typically presents as septicaemia, meningitis, or meningoencephalitis (Doganay, 2003; Vázquez-Boland *et al*., 2001).

Listeriosis is regularly reported in Europe and North America, but in Africa and other developing countries, Nigeria inclusive, where the food industries are not very developed, only a few sporadic cases have been reported (Boukadidda *et al*., 1994; David and Odeyemi, 2007).

Though the prevalence is low, 0.4-0.8/100 000 inhabitants, various outbreaks of listeriosis were pointed out in industrialized countries of Europe and USA, the most frequent being associated with dairy, vegetables and meat products consumption (Robert, 1994).

Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illnesses (Swaminathan and Gerner-Smidt, 2007). Reports show that *L. monocytogenes* infections are responsible for high hospitalization rates and have been linked to sporadic episodes and large outbreaks (Jemmi and Stephan, 2006; Warriner and Namvar, 2009).

The different conditions in which this pathogenic agent survives in the environment, the remarkable resistance in the processing area, the multiplication capacity at refrigeration temperature, the long persistence in food even in hostile conditions, make *L. monocytogenes* an important health threat for the population health status (Kozak *et al*., 1996). Furthermore, *L. monocytogenes* is of great economic relevance for food business operators because contamination of food products might result in large recalls and changed buying behaviour of consumers (CDC, 2002; Jemmi and Stephan, 2006). Amongst other foods, ready-to-eat products have recently often been implicated in recalls and outbreaks (CDC, 2002; Swaminathan and Gerner-Smidt, 2007). Thus, the ability of *L. monocytogenes* to persist in food-processing environments and to grow at low environmental temperatures present major challenges for the food industry and food safety (Tasara and Stephan, 2006; Warriner and Namvar, 2009).

1.2 Statement of Research Problem

Although, *L. monocytogenes* is widespread in the environment it is considered to be

intolerant to the temperature achieved during food processing, such as cooking and pasteurization. However, post processing contamination of products with strains associated with the processing environment can occur (Harvey and Gilmour, 1993). Furthermore various studies have indicated that certain strains of *L. monocytogenes* survive within the food processing environment (Senezek *et al.,* 2000) and the persistence of such strains in a food processing plant is of particular concern as they have the potential to act as a continual sources of contamination of the processed products (Sashara and Zottola, 1993).

L. monocytogenes has been widely observed in foods, environmental and clinical samples. Its detection and identification in foods traditionally involve culture methods on selective pre enrichment, enrichment and plating. This is followed by the characterization of *Listeria* spp. using colony morphology, sugar fermentation and haemolytic properties (Gasanov *et al.*, 2009; Paoli *et al*., 2005). Conventional microbiological methods are usually very sensitive and remain the gold standard as compared to other methods.. These methods are particularly important when the bacterial culture is needed as the end result from positive samples. Although a negative result can be confirmed in 3-4 days, the time for a positive result is usually 5-7 days from sample collection (Paoli *et al*., 2005). As it is usually not possible to hold food products for 7 days prior to distribution, the food industry desires faster methods for the detection of *L. monocytogenes.*

The detection of *L. monocytogenes* in foods is also hampered by the high population of competitive micro flora, the low levels of pathogen, and interferences of inhibitory food components (Norton, 2002). As a result, in the last few years there has been a notable development of new culture media for the improved detection of *L. monocytogenes* in foods

and efficient methods based on antibodies or molecular techniques have also been developed (Gasonov *et al*., 2005). These new tests possess equal sensitivity and allow testing to be completed within 48h.

However, most of these detection methods, such as the most common selective media are unable to distinguish the different *Listeria* spp. Within the genus *Listeria*, only *L. mononcytogenes* is of public health significance (Farber and Peterkin, 1991; Swaminathan, 2001) which necessitates employing of species specific analytical methodologies. Furthermore, not all *L. monocytogenes* strains are equally capable of causing disease in humans (McLauchlin, 1990; Farber and Peterkin, 1991). Of the 16 serovars of *L. monocytogenes*, only three i.e; 1/2a, 1/2b and 4b, cause more than 90% of the human cases (Gellin and Broome, 1989; Swaminathan, 2001). A molecular technique (Multiplex Polymerase chain reaction) has been developed to characterize the four major *L. mononcytogenes* serovars (1/2a, 1/2b,1/2c and 4b) isolated from food and patients into distinct groups (Doumith *et al*., 2004).

L. monocytogenes is also problematic due to its resistance to antibiotics. The first multi-resistant strain of *L. monocytogenes* was isolated in France in 1988 (Poyart -Salmeron *et al*., 1990), thereafter *L. monocytogenes* strains resistant to one or more antibiotics have since been isolated (Franco - Albuin *et al*., 1994; Charpentier *et al*., 1995).

An increase in the antibiotic resistance of *Listeria monocytogenes* has previously been reported (Peterkin *et al*., 1991). In northern Nigeria and north America, it was reported that most strains of the organism were sensitive to ampicillin, erythromycin and other common antibiotics. But surprisingly, the same research study reported that the organism resisted

cephalosporins, nitrofurantoin, tetracycline, chloramphenicol at *in vitro* levels (Onyemelukwe *et al*., 1993; Cherubin *et a*l., 1991; Adetunji and Adegoke, 2008). A multiple-antibiotic resistant environmental isolate of *Listeria monocytogenes* has been reported in western Nigeria (David and Odeyemi, 2007).

In Nigeria, Onyemelukwe *et al.* (1983) reported the first confirmed case of human listeriosis in northern Nigeria involving *L. monocytogenes*. Enurah *et al*. (1988) reported an outbreak of avian listeriosis in Jos, Plateau. Oni *et al*. (1989) isolated the organism from cattle and milk in Kaduna state and Chukwu (1995) reported the occurrence of *L. monocytogenes* in processed beef and other dairy products in Jos and its environs.

There is a dearth of information on the occurrence of listeriosis in northwestern Nigeria. Apart from the earlier works done by Oni *et al.* (1989) and Onyemelukwe *et al*. (1983), there are to our knowledge no recent information on the prevalence and antibiogram of *L. monocytogenes* in raw milk and dairy products especially. This work is intended to fill this gap in the epidemiology of *L. monocytogenes*.

1.3 Justification

Nigeria is presently going through a period in which the unorganized sector is fast becoming organized with different fast food outlets springing up across major cities and considerable emphasis placed on food hygiene ensured by government regulatory agencies like National Agency for Food and Drug Administration and Control (NAFDAC).

Milk is said to be one of the most important nutrients and protein dense food, because it is

an excellent source of nine essential nutrients and casein, a major milk protein. Dairy products like yoghurt, made from milk and their consumption play a significant role in the supply of important nutrients and protein required for good health (Singh and Prakash, 2008). Milk products are widely consumed and the demand for them has existed in many parts of Nigeria. However, there is an increasing demand by consumers for high quality natural food, free from preservatives and contaminating micro organisms.

Raw milk and milk products such as yoghurt have been cited as the principal source of *L. monocytogenes* transmission (Farber and Peterkin, 1991). Milk constitutes a complex ecosystem for various micro organisms. Contamination of milk and milk products, with pathogenic bacteria is largely due to processing, handling and unhygienic conditions (Dalu and Faresu, 1996).

L. monocytogenes has been shown to have a high cross contamination rate from unhygienic equipments to animals and/or humans (Norrung, 2000). Additionally it is indicated that *L. monocytogenes* can resist even UHT pasteurization process by infiltrating the somatic cells and/or leukocytes in the raw milk. Furthermore, it has been shown that *L. monocytogenes* can produce listeriolysin O (LLO) before it is inactivated and the toxins can exist in milk and its products even if the microorganism does not exist in the milk and its products (Low *et al*., 1992).

Available information on *L. monocytogenes* infection in Northern Nigeria dates back to early 1980s by the work of Onyemelukwe *et al*. (1983) in which they isolated *L. monocytogenes* from 19 patients with clinical conditions characterized by meningitis, meningoencephalitis, spontaneous peritonitis, septicaemia, pelvic infection and arthritis. All

isolates were type four serotypes. Reports have confirmed the presence of *L. monocytogenes* in milk and milk products processed in Nigeria including ice cream, fermented milk and wholesome butter (Adetunji *et al*., 2003).

Milk-borne disease outbreaks associated with *L. monocytogenes* have been reported in developed countries but limited information is available in most African countries, including Nigeria (WHO, 2002). In Nigeria, like other African countries where the HIV/AIDS is prevalent, listeriosis may be a silent killer among the immune compromised individuals who may depend on milk and milk by products for protein intake.

In western Nigeria, multiple- antibiotic resistance in environmental isolates of *L.monocytogenes* has been reported (David and Odeyemi, 2007). Onyemelukwe *et al*. (1983) and Adetunji and Adegoke (2008) also reported that the organism resisted cephalosporin, nitrofurantoin, tetracycline and chloramphenicol, at *in vitro* level.

However current information on the prevalence of *L. monocytogenes* in raw milk and yoghurt and their antibiotic susceptibility profiles in Zaria are unknown. Thus, it is imperative to know if the consumption of milk is safe and to what extent, as milk is an important source of nutrition for man and animals. Data obtained from antibiograms will be useful in the management of patients with listeriosis.

1.4 Aim

To determine the prevalence and antibiograms of *L. monocytogenes* in raw milk and yoghurt sold in Zaria, Kaduna State.

1.5 Specific Objectives

1. To determine the prevalence of *L. monocytogenes* in raw milk and yoghurt sold in Zaria, Kaduna State by carrying out biochemical identification of *L. monocytogenes* isolates including the use of Microgen^{TM} Listeria ID-System.
2. To determine the antibiotic sensitivity of *L. monocytogenes* in raw milk and yoghurt in study area.
3. To determine the carriage of virulence gene of *hyl A* among *L. monocytogenes* isolates.

1.6 Research Question

Do raw milk and yoghurt retailed in Zaria, Kaduna State, harbour pathogenic and antibiotic resistant *Listeria monocytogenes?*

CHAPTER TWO

LITERATURE REVIEW

2.1 History of The Genus *Listeria.*

The bacterial genus *Listeria* was first discovered about 100 years ago. The first documented case of *Listeria* was in 1924. In the late 1920s, two researchers independently identified *Listeria monocytogenes* from animal outbreaks. They proposed the genus *Listerella* in honor of surgeon and early antiseptic advocate Joseph Lister; however, that name was already in use for a slime mold and a protozoan. Eventually, the genus *Listeria* was proposed and accepted. The best known and most dangerous species, *Listeria monocytogenes*, was soon identified as the occasional cause of disease in animals and humans. From then on, the study of *Listeria* and listeriosis remained a relatively obscure medical backwater until the early 1980s, when a startling change occurred. Numbers of human cases of listeriosis suddenly began to increase sharply at this time. For example, in France the number of people affected by the disease in the 1970s was a steady average of 15 per year. But by 1987 the number had risen to 687. Similarly, in the USA, figures for 1967-69 show that there were 255 cases of listeriosis. But in 1987 alone, 1,700 cases were recorded. Similar rises were seen in the UK and in many other countries worldwide (Todar, 2008).

The first hint of a link with food came in Canada in 1981. An outbreak of listeriosis in Halifax, Nova Scotia involving 41 cases and 18 deaths, mostly in pregnant women and neonates, was epidemiologically linked to the consumption of coleslaw containing cabbage

that had been treated with *L. monocytogenes*-contaminated raw sheep manure. Since then, numbers of human cases of listeriosis suddenly began to increase sharply

Although the incidence of human listeriosis has fallen since the 1980's and in many countries is almost back to the levels of the 1970s, foodborne outbreaks still occur. For instance, in 2002, an outbreak in the USA linked to cooked poultry products affected at least 46 people, caused 11 deaths and resulted in the largest ever recall of meat products in US history. This illustrates that, although the food industry has devoted significant resources to developing and implementing effective controls against *Listeria* since the 1980s, the problem has not gone away.

More than a dozen serious *Listeria* outbreaks have been recorded, often involving fatalities and linked to a wide variety of foods, including cheese, cooked meats and pâtés, chocolate milk, butter, and smoked fish (Todar, 2008).

2.2 Taxanomy

Listeria species belong to the kingdom, Bacteria; Phylum, Firmicutes; Class, Bacilli; Order, Bacillales; Family, *Listeriaceae;* and Genus, *Listeria* (Todar, 2008). The genus *Listeria* was first included in a broad group of coryneform bacteria. Members of the genus *Listeria* are asporogenous, nonbranching, regular, short Gram positive rods (0.5 to 2µm by 0.4 to 0.5µm) that occur singly or in short chains. Filaments 6 to 20µm long may occur in older or rough cultures. The organisms are motile at 28^{o}C by means of one to five peritrichous

flagella. Colonies are small (1 to 2mm after 1 or 2 days of incubaton at 37^{o}C) smooth and blue gray on nutrient agar when examined under obliquely transmitted light but are greenish blue on chromogenic *Listeria* agar (CLA). Growth occurs between 3 and 45^{o}C but the optimum growth temperature is between 3 and 37^{o}C. The genus *Listeria* currently contains ten species: *L. fleischmannii, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. rocourtiae, L. seeligeri, L. weihenstephanensis* and *L. welshimeri.* (Lang Halter *et al*., 2013). Under the microscope, *Listeria* species appear as small, Gram-positive rods, which are sometimes arranged in short chains. In direct smears, they may be coccoid, so they can be mistaken for streptococci. Longer cells may resemble corynebacteria. Flagella are produced at room temperature, but not at 37 °C. Heamolytic activity on blood agar has been used as a marker to distinguish *L. monocytogenes* among other *Listeria* species, but it is not an absolutely definitive criterion. Further biochemical characterization may be necessary to distinguish between the different species of *Listeria* (Todar, 2008).

Over the years, only *L. monocytogenes* has been associated with human illness (Seafood, HACCP Alliance, 2007). *Listeria* strains are divided into serotypes on the basis of somatic (O) and flagellar (H) antigen. There are sixteen serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 5, 7, 6a,(4f) and 6b(4g) of *L. monocytogenes* and all can cause disease, but more than 90% of human isolates belong to serotypes:1/2a, 1/2b and 4b. *L.monocytogenes* serotype 4b strains are responsible for 33 to55% of sporadic human cases worldwide and for all major foodborne outbreaks in Europe and North America since the 1980s (Dharmarha and Vaishali, 2008).

2.3 Epidemiology of *Listeria monocytogenes*

Reservoirs of *Listeria monocytogenes* are present in the environment, human and animal populations. Transmission of the disease causing bacteria can occur through:

a) Foodborne- *Listeria monocytogenes* has been associated with foods such as unpasteurized milk, cheeses, ice cream, raw vegetables, raw and smoked sausages, raw and cooked poultry, all types of raw meats, and raw and smoked fish. *L. monocytogenes* introduced as a foodborne disease initially manifests itself as a gastrointestinal illness before spreading through the blood stream to affect the brain and the nervous system.

b) Person to person spread- *Listeria monocytogenes* is spread from person to person in nosocomial and nursery settings.

c) Direct inoculation- Direct contact with infectious material. This is more common in people with lesions in their hands and arms and when they come in contact with the environment that is infected with *Listeria monocytogenes.*

d) In utero/ parental transmission- *Listeria monocytogenes* can be transmitted to the unborn foetus through an infected mother during her pregnancy term (Silver, 1998).

The incubation period for listeriosis is variable and ranges from 3 to 70 days, with the median incubation period being three weeks (Bortolussi, 2008).

CDC (2014) estimates that approximately 1600 illnesses and 260 deaths due to listeriosis occur annually in the United States. In 2013, the average annual incidence of listeriosis in the United States was 0.26 cases per 100,000 individuals (CDC, 2014).

In 2012, 831 foodborne outbreaks were reported to CDC, of which 4 were confirmed outbreaks and 1 suspected outbreak of listeriosis in the United States (CDC, 2014). The largest listeriosis outbreak in U.S. history occurred in 2011, when 147 illnesses, 33 deaths, and 1 miscarriage occurred among residents of 28 states; the outbreak was associated with consumption of cantaloupe from a single farm (CDC, 2011).

2.4 Pathogenesis of Listeriosis

Listeria uses the cellular machinery to move around inside the host cell: It induces directed polymerization of actin by the *act*A transmembrane protein, thus pushing the bacterial cell around (Smith and Portnoy, 1997).

Listeria monocytogenes, for example, encodes virulence genes that are thermoregulated. The expression of virulence factor is optimal at 39 °C, and is controlled by a transcriptional activator, *prfA*, whose expression is thermoregulated by the *prfA* thermoregulator UTR element. At low temperatures, the *prfA* transcript is not translated due to structural elements near the ribosome binding site. As the bacteria infect the host, the temperature of the host melts the structure and allows translation initiation for the virulent genes (Todar, 2008)..

The majority of *Listeria* bacteria are targeted by the immune system before they are able to cause infection. Those that escape the immune system's initial response, however, spread through intracellular mechanisms and are, therefore, guarded against circulating immune factors (Todar, 2008).

To invade, *Listeria* induces macrophage phagocytic uptake by displaying D-galactose in their teichoic acids that are then bound by the macrophage's polysaccharide receptors. Other important adhesins are the internalins. Once phagocytosed, the bacterium is encapsulated by the host cell's acidic phagolysosome organelle (Southwick and Purich, 2007). *Listeria*, however, escapes the phagolysosome by lysing the vacuole's entire membrane with secreted *hemolysin* (Tinley *et al*., 1989), now characterized as the exotoxin listeriolysin O. The bacteria then replicate inside the host cell's cytoplasm (Todar, 2008).

Listeria must then navigate to the cell's periphery to spread the infection to other cells. Outside the body, *Listeria* has flagellar-driven motility, sometimes described as a "tumbling motility". However, at 37 °C, flagella cease to develop and the bacterium instead usurps the host cell's cytoskeleton to move (Laine *et al.*, 1998). *Listeria*, inventively, polymerizes an actin tail or "comet", from actin monomers in the host's cytoplasm with the promotion of virulence factor *Act*A. The comet forms in a polar manner and aids the bacteria's migration to the host cell's outer membrane. Gelsolin, an actin filament severing protein, localizes at the tail of *Listeria* and accelerates the bacterium's motility (Laine *et al.*, 1998). Once at the cell surface, the actin-propelled *Listeria* pushes against the cell's membrane to form protrusions called filopods or "rockets". The protrusions are guided by the cell's leading edge to contact adjacent cells, which then engulf the listeria rocket and the process is

repeated, perpetuating the infection (Galbraith *et al.,* 2007). Once phagocytosed, the bacterium is never again extracellular: it is an intracytoplasmic parasite (Tinley, 1989).

2.5 Virulence Factors in Listeria species

There are several virulence factors which are thought to be important in the pathogenesis of *Listeria*, these include a cluster of six genes (*prf*A, *plc*A, *hyl*A, *mpl*, *act*A *and plc*B) in a genomic element referred to as the *prfA* virulence cluster or the *Listeria* pathogenicity island (LiPl). Also involved in virulence are members of the internalin family (Schmid *et al*., 2005). Genes in the *prf*A cluster encodes functions that are necessary for inter and intracellular motility and intracellular survival in the host cell. Internalin genes encodes proteins essential for host cell invasion (e.g *inl*A and *inl*B) (Bierne *et al.,* 2007), *inl*C has recently being shown to encode a protein critical for cell to cell spread (Rajabian *et al.*, 2010) and the functions of a number of internalin proteins still remain to be illucidated (Milillo and Wiedmann, 2009). A number of internalin genes are also organized in clusters including the *inl*AB operon, *inl*GHE operon (which can also be present as an *inl*GC2DE or as an *inl*C2DE operon), which is found in *L. monocytogenes* and an *L. invanovii* specie specific pathogenicity island encoding sphingomyelinase and numerous internalins (Dominguez-Bernal *et al.,* 2006).

Importantly the presence or absence of the *prf*A cluster and virulence characteristics can also be used to classify *Listeria* species and clades into three groups, including (i) species that contain the *prf*A virulence cluster and are known pathogens like *L. monocytogenes and L. invanovii*, (ii) species that lack *prfA* virulence cluster and are non pathogenic e.g *L. marthii and L. welshimeri.* (iii) Species in which the presence of the *prfA* virulence cluster

varies by strain. The last group contains *L. seeligeri*, which is non pathogenic, although the majority of strains in the population contain the *prfA* virulence cluster (Volokhov *et al.*, 2007) and *L. innocua* which is also non pathogenic, and although most strains lack the *prfA* virulence cluster, a small proportion of strains do carry this cluster (Johnson *et al.*, 2004; Volokhov *et al*., 2007).

Studies indicated haemolysin to be the major virulence factor of *L. monocytogenes*; however, haemolysin alone cannot be used as an indicator of the presence of virulent specie because *L. seeligeri* is haemolytic but not pathogenic (Geoffrey *et al.*, 1987). The organism is capable of multiplication within cells of the monocyte macrophage series and is capable of entering the cell, escaping from the phagosome, multiplying within the cytoplasm and spreading between cells. Crucial to the virulence of the organism is its ability to escape intracellular killing within macrophages by lysis of phagosomal membrane and escape into the cytoplasm, which is mediated by its secretion of a haemolysin, listeriolysin O (LLO). The importance of LLO as a virulence determinant has been clearly established (Geoffrey *et al.,* 1987).

Several other virulence determinants have been identified which are involved or associated with bacterial survival. The majority of the genes involved are arranged around the haemolsin gene forming a virulence cluster which is also present in other haemolytic *Listeria* species (Gouin *et al*., 1994) the *prfA* positively regulates expression of LLO in pathogenic strains and encodes a protein of 27kDa molecular mass which has no significant homology to other bacterial regulatory proteins (Liemeister-Wachter *et al.,* 1990). Further studies have shown the *prf*A gene to regulate its own synthesis and to be be a pleo

trophic regulator of virulence determinants involved in invasion , intracellular bacterial motility and cell to cell spread (Chakraborty *et al.,* 1992). The phosphatidylinositol-specific phospholipase C gene encodes a protein of approximately 36kDa molecular mass (Camilly *et al*., 1991; Leimeister- Wachter, 1991) which may act in concert with LLO for lysis of the phagosomal membrane . However, mutations within the gene exert a polar effect upon the regulatory gene *prf*A and it is therefore, difficult to determine the true role of phosphatidylinositol- specific phospholipase C in virulence. Movement within the host cell cytoplasm follows encapsulation of the organism by actin filament which propels the bacterium into the neighboring cells (Mounier *et al.*, 1990). A gene termed *actA* included in the virulence cluster encodes a surface protein with a calculated molecular mass of 67kDa (Kocks *et al*., 1992) and although the protein's functions is obscure, it is clearly involved in actin assembly since strains having mutations within the *actA* gene do not polymerise actin and have markedly reduced virulence. The operon which incoperates the *actA* gene also includes two other virulence associated genes one of which encodes a 29kDa molecular mass, zinc dependent phospholipase possessing lecithinase activity (Geoffrey *et al*., 1991). This is apparently involved in lysis of the double cell membrane which forms a barrier to cell to cell spread. Immunoblotting of *L. monocytogenes* culture supernatant fluids has identified, in addition to the lecithinase, an antigenically related protein which is now recognised as the enzyme precursor to the mature 29kDa protein (Geoffrey *et al*., 1991).

Though these mechanisms of intracellular multiplication have been clarified, the means for bacterial adherence to cells are unknown. Entry of *L.monocytogenes* to both phagocytic and epithelial cells has been demonstrated *invitro* and is possibly associated with production of

an 80kDa molecular mass protein ‘internalin’. Sequencing of the internalin gene predicts a protein with a cytoplasmic membrane anchor and a structure reminiscent of *Streptococcus pyogenes* M protein (Kathariou *et al.,* 1990). The gene is part of a gene family regulated by *prfA* and under non stringent conditions; a gene probe has detected several related genes in *L.monocytogenes, L.invanovii, L. innocua.* A protein termed P60 may also have a role in invasion as rough forms of *L. monocytogenes* lacking P60 are unable to enter cells. However the protein is unrelated to internalin and there are contradictory reports of its requirements for cell invasion (Kathariou *et al.*, 1990). Monocytosis producing agent (MPA) has been extracted from *L. monocytogenes* and the failure of similar extracts from *L. innocua* to stimulate a monocytosis suggests MPA may have a role in virulence.

2.6 Listeriosis

Listeriosis is a bacterial infection most commonly caused by *Listeria monocytogenes* (Ryan and Ray, 2003), although, *L. ivanovii* and *L. grayi* have been reported in certain cases. Listeriosis primarily causes infections of the central nervous system (meningitis, meningoencephalitis, brain abscess, cerebritis) and bacteremia in those who are immunocompromised, pregnant women, and those at the extremes of age (newborns and the elderly), as well as gastroenteritis in healthy persons who have ingested a large inoculum of the organism (Dogany, 2003). *Listeria* is ubiquitous in the environment and is primarily transmitted via the oral route after ingestion of contaminated food products, after which the organism penetrates the intestinal tract to cause systemic infections. The diagnosis of listeriosis requires the isolation of the organism from the blood and/or the cerebrospinal fluid. Treatment includes prolonged administration of antibiotics, primarily

ampicillin and gentamicin, to which the organism is usually susceptible. The case fatality rate for those with a severe form of infection may approach 25% (Todar, 2008). Although, *Listeria monocytogenes* has low infectivity, it is hardy and can grow in temperatures from 4 °C (39.2 °F) (the temperature of a refrigerator), to 37 °C (98.6 °F), (the body's internal temperature). (Southwick and Purich, 2007). Listeriosis is a serious illness and manifest in different forms.

*2.6.*1 *Invasive Listeriosis*

Invasive listeriosis typically has a 2 to 3 week incubation time, but can sometimes extend up to three months (Gellin and Broome, 1989). Serious conditions caused by *Listeria monocytogenes* in adults can include septicaemia, meningitis, enceplalitis, abortion, or stillbirth (Shelef, 1989). Invasive diseases in nonpregenant adults can include a variety of other clinical manifestations. Endocarditis can occur in patients with underlying cardiac lesions. Cutaneous infections have been reported in persons handling animals and those exposed by accidental exposure while working in laboratories. Focal infections are rare but can include endophthalmitis, septic arthritis, osteomyelitis, pleural infection and peritonitis (Slutsker and Schuchat, 1999).

The probability of tissue invasion depends upon the number of organisms consumed, host susceptibility, and virulence of the strain (Gellin and Broome, 1989). Most cases of listeriosis occur in foetuses or neonates and individuals with a predisposing condition that impairs the immune system (Slutsker and Schuchat, 1999).

2.6.2 Non-Invasive Listeriosis (Listerial Gastroenteritis)

Gastrointestinal illness (listerial gastroenteritis) from *Listeria monocytogenes* has been recognized as a distinct entity (Dalton *et al*., 1997). Typical signs and symptoms associated with the mild form of *Listeria monocytogenes* infection are primarily those associated with gastrointestinal illness: chills, diarrhoea, headache, abdominal pain and cramps, nausea, vomiting, fatigue, and myalgia. A variety of foods such as milk, ice cream, yoghurt have been implicated as the vehicles of infection (Adetunji and Arigbede, 2011)). Because symptoms are mild, there is a high potential for underreporting of listerial gastroenteritis. Data are currently unavailable through foodborne surveillance mechanisms such as FoodNet to capture the incidence of listerial gastroenteritis since routine stool cultures do not include evaluation for *Listeria monocytogenes*.

Nevertheless, outbreaks of listerial gastroenteritis have been identified, most of the cases reported mild symptoms (Dalton *et al.*, 1997; Aureli *et al.*, 2000). In the vast majority of these cases, there was no evidence for invasive disease beyond the intestine. Gastrointestinal and other mild symptoms were reported in individuals with no known underlying predisposition. In two of these reports, there was evidence of very high levels of food contamination. These facts suggest that, in normal individuals, listerial gastroenteritis may be associated with exposure to high levels of *Listeria monocytogenes*. It is possible that this manifestation of *Listeria monocytogenes* infection is a different disease compared to invasive and more severe listeriosis. Because modeling in this risk assessment depends on case reporting and non-invasive gastroenteritis is not likely to be reported, listerial gastroenteritis was not considered in the risk assessment model. However, the outbreaks do

provide important observations related to the exposure of populations to extremely high levels of the microorganisms without identifiable cases of invasive listeriosis.

2.6.3 Asymptomatic Carriage

The large intestine may be a reservoir for *Listeria monocytogenes* in humans. Estimates of faecal carriage in various populations of healthy adults range from <1% to 21%. It has been suggested that stress can undermine resistance in faecal carriers, and may trigger listeriosis in the carrier (Mascola *et al.,* 1992). Several studies have looked at fecal carriage to gain insight into listeriosis. However, it is unknown how faecal carriage relates to length of incubation or occurrence of invasive disease (Mascola *et al.,* 1992; and Schuchat *et al.,* 1991).

Approximately 1 to 5% of normal asymptomatic carriers shed *Listeria monocytogenes* bacteria in the faeces (Hof, 2001). *Listeria monocytogenes* was isolated from 2 of 100 colon biopsy specimens from patients with colon cancer; however, neither patient exhibited signs of listeriosis (Hof, 2001).

In a retrospective study of the outbreak in 1985 that was linked to Hispanic-style fresh soft cheese, outbreak-related listeriosis patients and matched controls were asked to participate in a study of stool carriage of *Listeria monocytogenes* (Mascola *et al*., 1992). Faecal carriage incidence was also determined for employees of the cheese plant and their household contacts. Stool specimens from 8% of those tested were positive for *Listeria*

monocytogenes. The highest rate of recovery of the organism from stool samples was from employees of the cheese plant and their household contacts. It was found that the occurrence of listerial gastroenteritis or listeriosis was not associated with fecal carriage of *Listeria monocytogenes,* and was actually more common for persons with negative stool samples (Mascola *et al.*,1992).

Between January 1990 and December 1991, as part of a multistate active surveillance project on sporadic listeriosis, a study was conducted to evaluate the faecal carriage of *Listeria monocytogenes* among household contacts of patients with invasive listeriosis (Schuchat *et al.*, 1993). The authors determined that the rates of carriage did not vary significantly by sex but were significantly higher in younger persons. The organism was isolated from 32% of those <30 years of age, compared to 7% from older persons. Nearly 20% of household contacts of patients with sporadic listeriosis had asymptomatic carriage of the strain associated with illness. The authors suggested that carriage of *Listeria monocytogenes* is more common in persons that have been in contact with listeriosis patients and that it was difficult to compare the faecal carriage rate in this study group to the population at large.

Listeriosis in human is caused by a facultative intracellular bacterium; *Listeria monocytogenes.* It is one of the most virulent food borne pathogens with 20 to 30% of clinical infections resulting in death (Ramaswamy *et al*., 2007). It is responsible for approximately 2,500 illnesses and 500 deaths in the United States annually. Listeriosis in man is the leading cause of death among food borne bacterial pathogens with fatality rates exceeding even *Salmonella* and *Clostridium botulinum* (Dharmarha, 2008). Studies suggest

that upto 10% of human gastrointestinal tract may be colonized by *L. monocytogenes* (Ramaswamy *et al.,* 2007). Also, the presence of this bacterium in intestinal tract of 5 to 10% of healthy humans without any obvious symptoms of the disease, was established (Todar, 2009*).*However, in healthy adult individuals it can be totally unnoticed or be illness like the flu.

Nevertheless clinical diseases due to *L. monocytogenes* are more frequently recognised by Veterinarians, especially as meningo-encephalitis in ruminants. It is the third most common cause of meningitis in newborns.

Several segments of the population are at increased risk and need to be informed so that proper precautions can be taken (Mayo Clinic, 2009). The body's defense against *Listeria* is by "cell-mediated immunity" (Cossart and Bierne, 2001). Therefore, individuals whose cell-mediated immunity is suppressed are more susceptible to the devastating effects of listeriosis, including especially HIV-infected individuals, who have been found to have a *Listeria*-related mortality of 29%. Pregnant women naturally have a depressed cell-mediated immune system (Silver, 1998). In addition, the immune systems of foetuses and newborns are very immature and are extremely susceptible to these types of infections. (Silver,1998). Other adults, especially transplant recipients and lymphoma patients, are given necessary therapies with the specific intent of suppressing T-cells, and these individuals become especially susceptible to *Listeria* as well (CDC, 2011).

According to the CDC and other public health organizations, individuals at increased risk for being infected and becoming seriously ill with *Listeria* include the following groups:

- Pregnant women: They are about 20 times more likely than other healthy adults to get listeriosis. About one-third of listeriosis cases happen during pregnancy.
- Newborns: Newborns rather than the pregnant women themselves suffer the serious effects of infection in pregnancy.
- Persons with weakened immune systems
- Persons with cancer, diabetes, or kidney disease
- Persons with AIDS: they are almost 300 times more likely to get listeriosis than people with normal immune systems.
- Persons who take glucocorticosteroid medications (such as cortisone)
- The elderly

A person with listeriosis usually has fever and muscle aches, sometimes preceded by diarrhoea or other gastrointestinal symptoms. Almost everyone who is diagnosed with listeriosis has "invasive" infection, in which the bacteria spread beyond the gastrointestinal tract. The symptoms vary with the infected person:

- Pregnant women: Pregnant women typically experience fever and other non-specific symptoms, such as fatigue and aches. However, infections during pregnancy can lead to miscarriage, stillbirth, premature delivery, or life-threatening infection of the newborn.
- People other than pregnant women: Symptoms can include headache, stiff neck, confusion, loss of balance, and convulsions in addition to fever and muscle aches (CDC, 2011).

Listeriosis can present in different ways. In older adults and people with immune-compromising conditions, septicemia and meningitis are the most common clinical presentations. Pregnant women may experience a fever and other non-specific symptoms, such as fatigue and aches, followed by feotal loss or bacteremia and meningitis in their newborns. Immunocompetent people may experience acute febrile gastroenteritis or no symptoms (CDC, 2011).

The sequelae of listeriosis in persons, who suffer a *Listeria* infection that does not resolve on its own, can be many (Bortolussi, 2008). The most common is septicemia (bacterial pathogens in the blood, also known as bacteremia), with meningitis being the second most common. Other complications can include inflammation of the brain or brain stem (encephalitis), brain abscess, inflammation of the heart-membrane (endocarditis), and localized infection, either internally or of the skin.

Death is the most severe consequence of listeriosis, and it is tragically common (Bennion *et al*., 2008). For example, based on 2009 FoodNet surveillance data, 89.2% of *Listeria* patients ended up in the hospital, the highest hospitalization rate for pathogenic bacterial infection In persons 50 years of age and older, there was a 17.5% fatality rate—also the highest relative to other pathogens (CDC, 2010).

2.7 Listeriosis in Animals

Animal listeriosis is an infectious but not contagious disease caused by the bacterium *Listeria monocytogenes*, far more common in domestics animals (domestic mammals and

poultry), especially ruminants. It can also occur in feral animals among others, game animals as well as in poultry and other birds (Merck Veterinary Manual, 2010).

The causative bacterium lives in the soil and in poorly made silage and is acquired by ingestion. It is not contagious; over the course of 30-year observation period of sheep disease in Morocco, the disease only appeared in the late 2000s when feeding bag-ensiled corn became common. In Iceland, the disease is called "silage sickness". In sheep, the disease is also called the "circling disease"(Merck Veterinary Manual, 2010).

The disease is usually sporadic, but can occur as farm outbreaks in ruminants. Listeriosis is primarily a winter-spring disease of feedlot or housed ruminants. The less acidic pH of spoiled silage enhances multiplication of *L monocytogenes*. Outbreaks typically occur ≥10 days after feeding poor-quality silage. Removal or change of silage in the ration often stops the spread of listeriosis; feeding the same silage months later may result in new cases.

Three main forms are usually recognized throughout the affected species:

- encephalitis, the most common form in ruminants
- late abortion
- gastro-intestinal septicaemia with liver damage, in monogastric species as well as in preruminant calves and lambs

The various manifestations of infection occur in all susceptible species and are associated with characteristic clinical syndromes: abortion and perinatal mortality in all species, encephalitis or meningoencephalitis in adult ruminants, septicemia in neonatal ruminants

and monogastric animals, and septicemia with myocardial or hepatic necrosis (or both) in poultry.

Listeric encephalitis affects sheep, cattle, goats, and occasionally pigs. It is essentially a localized infection of the brain stem that develops when *L monocytogenes* ascends the trigeminal nerve. Clinical signs vary according to the function of damaged neurons but often are unilateral and include depression (ascending reticular activating system), ipsilateral weakness (long tracts), trigeminal and facial nerve paralysis, and less commonly, circling (vestibulocochlear nucleus). Neurologic signs indicating bilateral cranial nerve deficits are occasionally seen in lambs <4 mo old.

Septicemic or visceral listeriosis is most common in monogastric animals, including pigs, dogs, cats, domestic and wild rabbits, and many other small mammals. These animals may play a role in transmission of *L monocytogenes*. This form is also found in young ruminants before the rumen is functional. Although rare, septicemia has been reported in older domestic ruminants and deer. The septicemic form affects organs other than the brain, the principal lesion being focal hepatic necrosis. (Merck Veterinary Manual, 2010).

The uterus of all domestic animals, especially ruminants, is susceptible to infection with *L monocytogenes* at all stages of pregnancy, which can result in placentitis, foetal infection and death, abortion, stillbirths, neonatal deaths, metritis, and possibly viable carriers. The metritis has little or no effect on subsequent reproduction; however, *Listeria* may be shed for ≥1 month via the vagina and milk.

Infections acquired via ingestion tend to localize in the intestinal wall and result in prolonged fecal excretion. It has been postulated that contaminated silage results in latent infections, often approaching 100% of the exposed herd or flock, but clinical listeriosis occurs in only a few animals.

Listeriosis is relatively uncommon in pigs, with septicemia occurring in those <1 month old and encephalitis in older pigs; it has a rapid, fatal course of 3–4 days.

L. invanovii is also recorded as a cause of abortion in sheep and cattle but occurs less frequently than *L.monocytogenes* (Radostits *et al.*, 1994).

Listeriosis in animals can rarely be cured with antibiotics (tetracyclines, chloramphenicol and benzyl penicillin also) when diagnosed early, in goats, for example, by treating upon first noticing the disease's characteristic expression in the animal's face, but is generally fatal (Radostits *et al.*, 1994).

2.8 Occurrence of *Listeria* spp in Food

Listeriosis can occur sporadically or epidemically, but in both patterns, contaminated foods are the primary vehicles of transmission. Foods implicated as vehicles of infection include coleslaw (cabbage), soft cheeses, pâté poultry, turkey frankfurters, mushrooms, milk and pork tongue in jelly. One outbreak of gastroenteritis with fever but without progression to invasive disease was linked to consumption of milk that was highly contaminated by *L. monocytogenes (*Dalton *et al.*, 1997).

L. monocytogenes has been isolated from various ready-to-eat products. In a study by Meldrum *et al.* (2010) the prevalence of *L. monocytogenes* was found to be 4.1% in crustaceans (n=147), 6.7% in smoked fish (n=178), 2% in sushi (n=50) and 0.9% in green salad (n=335) samples in Wales. Wong *et al*. (2005) isolated *L. monocytogenes* from 1% of ham (n=104) and 1.7% of pate (n=60) samples in New Zealand. *L. monocytogenes* has also been isolated from dairy products. For example, *L. monocytogenes* was detected in 1.3% of fresh cheese samples in Spain (n=78), 0.2% of hard cheese samples in the United Kingdom (n=1242) and 0.3% of ice creams in Italy (n=1734) (Little *et al.*, 2009). The prevalence of *L. monocytogenes* in bulk milk tank internationally is estimated to be in the range of 1–60% (FSANZ, 2009).

Ready-to-eats foods have been found to be a notable and consistent source of *Listeria*. The presence of *L. monocytogenes* in ready-to-eat products is probably due to contamination occurring after the product has been processed. This contamination may occur during additional handling steps such as peeling, slicing and repackaging. Also, in the retail and food service environment, contamination may be transferred between ready-to-eat products (Lianou and Sofos, 2007). The type of handling that ready-to-eat meat receives may also influence the level of *L. monocytogenes* contamination. In a survey of retail packaged meats, there was a significantly higher prevalence of *L. monocytogenes* reported in products cut into cubes (61.5%) (n=13), compared with sliced products (4.6%) (n=196) (Angelidis and Koutsoumanis, 2006).

Ready-to-eats foods have been found to be a notable and consistent source of *Listeria*. For example, a research study done by the *Listeria* Study Group (Pinner *et al.*, 1992), found that *Listeria monocytogenes* was isolated from at least one food specimen in the refrigerators of 64% of persons with a confirmed *Listeria* infection (79 of 123 patients), and in 11% of more than 2000 food specimens collected in the study. Moreover, 33% of refrigerators (26 of 79) contained foods that grew the same strain with which the individual had been infected, a frequency much higher than would be expected by chance (Pinner *et al.*, 1992).

A widely cited USDA study that reviewed the available literature also summarized that: in samples of uncooked meat and poultry from seven countries, up to 70 percent had detectable levels of *Listeria* (USDA-FSIS, 2013).

Schuchat *et al*. (1991) found that 32 percent of the 165 culture-confirmed listeriosis cases could be attributed to eating food purchased from store delicatessen counters or soft cheeses.

2.9 Presence of *L. monocytogenes* in Milk and Dairy Products

In dairy industry, occasional presence of bacteria *L. monocytogenes* in milk, dairy products and dairy facilities/plant, is a major problem. Pasteurization of milk destroys *L. monocytogenes*. However, to which extent the *L. monocytogenes* is destroyed in milk during the process of pasteurization depends on the resistance of individual strains within the same species (Kasalica *et al.*, 2011).

Pasteurization of milk which occurs at the temperature of 62.8^0C for 30 minutes and 71.7^0C for 15 seconds is adequate to destroy *Listeria* present in the population of 102 cfu/ml, but not in the population of 107 cfu/ml (Jayamanne and Samarajeewa, 2010). According to research by Pearson and Marth (1990), high temperature short time pasteurization inactivates *L. monocytogenes,* but the minimum survival of the bacteria is still possible. *L. monocytogenes has* the ability to reproduce at very low storage temperatures (4^0C) (Kasalica and Otenhajmer, 1995a; 1995b; 1995c; 1996).

According to many authors, *L. monocytogenes* is most commonly isolated from raw milk sampled from collection tanks on farms or in dairy plants, and various contamination degrees have been recorded. In some countries the percentage of contaminated samples was relatively high, which speaks of potential danger of listeriosis if such milk is consumed without prior heat treatment (Kasalica *et al.*, 2011).

Table 2.1: Presence of *Listeria monocytogenes* in raw milk

Country	**Sampling location**	**Presence of *L.monocytogenes(%)***	**Reference**
Estonia	Collection tank of the farm and dairy plant	37	Haekkinen *et al.*, (2001).
Scotland	Collection tank of the Farm	15.6	Jay *et al.*, (2005).
Uganda	Collection tank of the dairy plant	13.0	Mugampoza *et al.*, (2011).
The Netherlands	Raw milk	4.38	Beckers *et al.*, (1987).
Sweden	Silo tank of the dairy plant Collection tank of the Farm	19.6 1.0	Waak *et al.*, (2002).
USA	Raw milk	4.0	Pearson & Mart, (1990).
Iran	Raw milk in dairy plant	1.7 – 3.3	Mahmoodi, (2010).
Turkey	Raw milk	1.17	Tasci *et al.*, (2010).

Source: Kasalica *et al*., 2011.

Presence of *L. monocytogenes* in cheese can be associated with type of cheese, the manufacturing process, inadequate pasteurization, post-pasteurization contamination, ability to multiply during storage at low temperatures and resistance to sanitation preparations (Bottarelli *et al., 1999).* The composition of cheese, pH, % of moisture, % of salt, ripeness of cheese, storing conditions, starter cultures and virulence of pathogens influence the reproduction of *L. monocytogenes* in cheese (Kovinčić *et al., 1991).* According to research conducted by many authors, cheeses produced from raw milk are more often contaminated with *L. monocytogenes,* compared to cheeses obtained from pasteurized milk. In Sweden, in samples (333) of soft and semi-soft cheeses collected from retail stores, *L. monocytogenes* was isolated in 6% of samples, of which 42% were produced from raw milk, and 2% from heat treated milk (Rudolf and Scherer, *2001).*

In a research carried out by McLauchlin *et al.* (1990), soft cheeses, immediately after production, had low presence of *L. monocytogenes* (<10/g). However, after the expiration date, their presence was significantly greater (105-107cfu/g), indicating how these cheeses are suitable for development and growth of *L. monocytogenes.* In semi-hard cheeses, in later stages of ripening, the pH value increases which is also suitable for growth of *L. monocytogenes*. Hard cheeses, because of low water content, are not the most suitable environment for growth of this bacterium.

Lactic acid bacteria (LAB) slow down or stop the growth of pathogens in fermented products (Kasalica, 1997*).* *L. monocytogenes* is characterized by different levels of sensitivity to LAB. In fermented products, *L. monocytogenes* has the ability to survive

during production process and storing. It survives in yoghurt for several weeks depending on the degree of contamination by this bacterium (Zuniga-Estrada *et al.*, 1995*)*. In dairy products fermented by using different LAB strains fermentation of milk (*L. bulgaricus, S. thermophilus*), the survival of *L. monocytogenes* depended on the strain of LAB and it ranged from 1-12 days (in yoghurt) to 4-37 weeks (milk fermented with *S. thermophilus*) (Schaack and Marth, 1988). The survival of *L. monocytogenes* in yoghurt, during storage at 4^0C, was influenced by pH value and dry matter of the yoghurt, and content of fat had no significant effect. Namely, all tested strains of *L. monocytogenes* survived longer in skimmed milk with high content of dry matter and high pH value compared to full fat milk with lower content of dry matter (Griffit and Deibel, 1990).

L. monocytogenes has also been isolated from ice cream and frozen food, which represents another proof that this microorganism can survive freezing temperatures (Cordano and Rocourt, 2001). According to literature, the frequency of isolation of *L. monocytogenes* in ice-cream ranges from 2.8 - 3.5% (Molla *et al.*, 2004). However, according to some researches, the rate may even be higher. In Addis Ababa, *L. monocytogenes* was isolated from 19.6% of ice-cream samples collected from September 2003 to April 2004. Increased contamination of ice-cream was explained by very suitable environment for its growth, considering the pH value, water activity, availability of nutrients and storage temperature (Molla *et al.*, 2004).

2.10 Effects of environmental factors on the Growth of *Listeria monocytogenes.*

(i) Temperature

Listeria monocytogenes is psychrotrophic and this is the reason why it is a particular risk in extended shelf life chilled foods. Extremely slow growth of *L. monocytogenes* has been recorded at temperatures as low as -1.5ºC and the maximum temperature for growth is generally accepted as 45ºC (Kasalica *et al.,* 2011). The organism survives well in frozen foods, but survival times can be shortened under acid conditions.

Although *Listeria monocytogenes* is not particularly heat resistant, it is more heat resistant than some other foodborne pathogens, such as *Salmonella* and *E. coli* O157:H7. It is readily inactivated at temperatures above 70ºC and heat processes such as commercial milk pasteurisation will destroy numbers typically found in milk (Kasalica *et al.,* 2011). Typical values in food substrates are between 5-8 minutes at 60ºC, and 0.1-0.3 minutes at 70ºC. Concern about the pathogen in particular food product categories has lead to heating guidelines being issued by various health authorities. The UK Department of Health advised that ready to eat meals or similar products should receive a heat treatment of at least 2 min at 70ºC, or equivalent, sometimes referred to as a *'Listeria* cook', to ensure the destruction of *L. monocytogenes*. For consumers, terms such as heat till 'piping hot' in the UK, and 'steaming hot' in the USA are used to describe safe heating regimes for certain foods (FAO/WHO, 2004)).

(ii) pH

The pH range for the growth of *L. monocytogenes* is 4.3 – 9.4 under otherwise ideal conditions, but the minimum pH is likely to be higher in real foods and at low temperatures. However, *L. monocytogenes* can survive for extended periods in acid conditions, particularly in refrigerated foods. *L. monocytogenes* reproduces/multiplies best at pH 7**.**0 It is resistant to acid (pH< 5) and alkali environment (kasalica *et al.*, 2011).

(iii) Water activity

The minimum water activity for the growth of *L. monocytogenes* is 0.92. *L. monocytogenes* is also able to survive for some time in low water activity environments, and may survive food drying processes. Survival times are extended at chilled temperatures (Tienungoon *et al.*, 2000).

(iv). Atmospheric Oxygen

Listeria monocytogenes grows well in the presence or absence of oxygen. Its growth is unaffected by many modified atmospheric conditions even at low temperatures. High concentrations of carbon dioxide are necessary to inhibit growth (Food Safety Watch, 2013).

(v) Chemicals

Although *Listeria monocytogenes* is not especially resistant to antimicrobials, it can prove difficult to control on food contact surfaces such as stainless steel because the bacteria can form persistent biofilms (Food Safety Watch, 2013).

2.11 Prevalence of *Listeria monocytogenes*

Navratilova *et al.* (2004) found a low prevalence (2.1%) of *L. monocytogenes* when in bulk milk samples. A similar frequency of *L. monocytogenes* in bulk tank milk samples has been reported from countries such as USA 4.1% (Rohrbach *et al.*, 1992) and Canada 1.9% (Fedio and Jackson, 1990).

Boujemaa *et al.* (2013) recorded a prevalence of 8.33%, 5.20% and 4.16% respectively for raw milk, traditionally fermented skimmed milk and traditionally soft white cheese commercialized in central north of Morrocco. Boubendir *et al.* (2011) reported 5.76% prevalence of *L. monocytogenes* in raw bovine milk produced in North Eastern Algeria. Similar results was found by Guerra *et al*. (2001), in Portugal in milk and dairy products to be 5%. Gaya *et al.* (1998) reported a low incidence of 3.6% of *L. monocytogenes* in raw milk produced in Spain, whereas in China, the prevalence of *L. monocytogenes* in raw milk was found to be very low (0.23 to 1.2%) (Ning *et al.*, 2013).

Among dairy products, yoghurt has received the least attention due to the fact that, its high acidity and milk pasteurization process before addition of starter, are effective barriers to the growth of pathogens including *L. monocytogenes* (Liu and Puri, 2008). The survival of

L. monocytogenes in yoghurt depends on its acidity and this bacterium disappears when the pH falls to 3.5 (Vermeulen *et al*., 2007).

2.12 Antibiotics Susceptibility of *Listeria monocytogenes*

The pattern of antibiotics susceptibility and resistance of *L. monocytogenes* has been relatively stable for many years (Charpentier, 1995). *In vitro*, the organism is susceptible to penicillin, ampicillin, gentamicin erythromycin, tetracycline, rifampicin and chloramphenicol but only moderately susceptible to quinolones. However many of these antimicrobial agents are bacteriostatic. Penicillin or ampicillin with or without an aminoglycoside is usually recommended for the treatment of listeriosis (Charpentier *et al.*, 1995). In cases of allergy to ß-Iactam antibiotics, trimethoprim (alone or in combination with sulfamethoxazole), tetracycline, erythromycin, and gentamicin constitute alternatives (Charpentier, 1995).

Studies *in vitro* and in animal models have shown that an aminoglycoside enhances the antimicrobial (bactericidal) activity of penicillin against *L. monocytogenes.* Trimethoprin – sulphamethoxaxole and aminoglycosides are among the few anti infective agents that are bactericidal to *L. monocytogenes*; only trimethoprim–sulphamethoxazole has been used occasionally with success (Aarestrup, 2012).

Listeria spp. are often exposed to low levels of antibiotics, as these agents are used in large amounts both in human and animal medicine (Aarestrup, 2012). This can lead to an

increased selective pressure which favors emergence of antibiotics resistance due to mutations or acquisition of mobile genetic elements. Thus, it was not surprising, when the first acquired antibiotics resistance in *L. monocytogenes* was reported in 1990 (Poyart-Salmeron *et al.*, 1990). Resistance plasmids conferring resistance to chloramphenicol, macrolides, and tetracyclines have been found in several clinical isolates of *L. monocytogenes a*nd have raised concern for the future (Poyart-Salmeron *et al.*, 1990).

Cephalosporins are ineffective *in vitro* and should never be administered when listeriosis is suspected (Hof *et al.*, 1997).

2.13 Preventive Measures for Listeriosis

The general guidelines recommended for the prevention of listeriosis are similar to those used to help prevent other foodborne illnesses, such as salmonellosis. In addition, there are specific recommendations for persons at higher risk for listeriosis (CDC, 2013).

General recommendations to prevent an infection with *Listeria*:

- Rinse raw produce, such as fruits and vegetables, thoroughly under running tap water before eating, cutting, or cooking. Even if the produce will be peeled, it should still be washed first.
- Separate uncooked meats and poultry from vegetables, cooked foods, and ready-to-eat foods.

- Be aware that *Listeria monocytogenes* can grow in foods in the refrigerator. Use an appliance thermometer, such as a refrigerator thermometer, to check the temperature inside the refrigerator. The refrigerator should be 40°F or lower and the freezer 0°F or lower.
- Clean up all spills in the refrigerator right away–especially juices from hot dogs and lunch meat packages, raw meat, and raw poultry.
- Clean the inside walls and shelves of your refrigerator with hot water and liquid soap, then rinse.
- Thoroughly cook raw food from animal sources, such as beef, pork, or poultry to a safe internal temperature.
- Use precooked or ready-to-eat food as soon as you can. Do not store the product in the refrigerator beyond the" use-by" date.
- Hot Dogs – store opened package no longer than 1 week and unopened package no longer than 2 weeks in the refrigerator.
- Luncheon and Deli Meat – store factory-sealed, unopened package no longer than 2 weeks. Store opened packages and meat sliced at a local deli no longer than 3 to 5 days in the refrigerator.
- Divide leftovers into shallow containers to promote rapid, even cooling. Cover with airtight lids or enclose in plastic wrap or aluminum foil. Use leftovers within 3 to 4 days.
- Do not drink raw or unpasteurized milk and do not eat foods that contain unpasteurized milk.

Recommendations for persons at higher risk, such as pregnant women, persons with weakened immune systems, and older adults in addition to the recommendations listed above, include:

- Avoiding eating hot dogs, luncheon meats, cold cuts, other deli meats (e.g., bologna), or fermented or dry sausages unless they are heated to an internal temperature of 165°F or until steaming hot just before serving.
- Avoid getting fluid from hot dog and lunch meat packages on other foods, utensils, and food preparation surfaces, and wash hands after handling hot dogs, luncheon meats, and deli meats.
- . Foods that do not need refrigeration, like canned or shelf-stable pâté and meat spreads, tuna, salmon and other fish products are safe to eat. Refrigerate after opening.

- Do not eat soft cheese, unless it is labeled as made with pasteurized milk. Make sure the label says, "MADE WITH PASTEURIZED MILK."

2.14 Control of *Listeria monocytogenes* in Foods.

The control of *Listeria* in foods relies largely on a HACCP approach and the establishment of effective critical control points (Food Safety Watch, 2013).

For food processors;

The careful design and layout of processing equipment in conjunction with regular, thorough cleaning of the processing environment can significantly reduce the level of *Listeria* contamination in many processed foods. However, because of its ubiquitous nature it is virtually impossible to eliminate the pathogen from many food products. Effective cooking processes should inactivate *Listeria* and its presence in cooked products can indicate poor hygiene during manufacture.

Other critical controls include strict temperature control, the prevention of cross-contamination between raw and processed foods and between the processing environment and processed foods, as well as the use of a restricted shelf life for potentially contaminated products that could support the growth of *Listeria* (FAO/WHO, 2004).

For retailers and consumers;

Appropriate scientifically-based methods should be used to devise safe shelf lives for 'at risk' chilled foods and these restricted shelf lives should be rigorously implemented and adhered to in order to reduce the risk from *L. monocytogenes*. Effective temperature control during distribution and storage is also a key to safe shelf life. Clear cooking instructions are needed on the packaging of chilled foods requiring reheating prior to consumption, to ensure that all parts of the product reach the temperature required to destroy the bacteria (Health Canada, 2011).

Vulnerable individuals, especially pregnant women, the elderly and the immunosuppressed are advised to avoid eating specific foods to reduce the risk from listeriosis. Health authorities in the UK advise these groups not to eat soft mould-ripened or blue-veined

cheeses, all types of pâté (including vegetable) and unpasteurised dairy products. These groups are advised that they may also choose to avoid cold (pre-cooked) meats and smoked salmon, and that they should thoroughly wash pre-packed salads and adequately heat chilled meals before eating. In the US the FDA also includes hot dogs, luncheon meats, cold cuts and smoked seafood (unless thoroughly reheated) to the list of foods that at-risk consumers should definitely avoid (US-FDA, 2013).

2.15 Immune Response to Listeriosis

The Gram-positive facultative intracellular bacterium *Listeria monocytogenes* is a model pathogen for elucidating important mechanisms of the immune response. Infection of mice with a sub-lethal dose of bacteria generates highly reproducible innate and adaptive immune responses, resulting in clearance of the bacteria and resistance to subsequent *L. monocytogenes* infection. Both the innate and adaptive immune systems are crucial to the recognition and elimination of this pathogen from the host (Lauren and Hao, 2007).

Since the early 1960s Mackaness demonstrated that cellular immunity was critical for control of infection in mice (Mackaness, 1962). In this model, bacteria are intravenously injected into the bloodstream of mice. Within minutes, most bacteria can be found in the spleen and liver where they are quickly internalized by resident macrophages (Conlan, 1996). In a sub-lethal infection, bacteria replicate until their numbers are controlled by activated macrophages. The development of a *Listeria*-specific T cell response is necessary to eliminate the bacteria and memory T cells provide protection to reinfection (Bhardwaj *et*

al., 1998). The generation of this protective immunity to rechallenge is dependent on immunization with live *L. monocytogenes*, capable of escaping into the cytosol. Immunization with heat-killed bacteria or bacteria deficient in the key virulence factor listeriolysin O (LLO), which mediates bacterial escape from phagosomes before degradation, does not lead to protective immunity (Bhardwaj *et al.*, 1998).

Innate immune responses are essential for early control of *L. monocytogenes* infection. Upon infection in the murine spleen, *L. monocytogenes* first localizes within macrophages in the marginal zone between the T cell rich white pulp and the B cell rich red pulp (Conlan, 1996). These infected cells then migrate into the white pulp region and form the beginning of a focus of infection that expands as neighboring cells become infected by the intercellular spread of bacteria. The innate immune response plays an important role in controlling bacterial growth and dissemination, preventing the spread into systemic, lethal infection (Conlan, 1996).

Although innate immune cells are important for initial control of *L. monocytogenes* infection, T cells are needed for final clearance of bacteria. CD4 and CD8 T cells are important for conferring sterilizing immunity (Bhardwaj *et al*., 1998).

Due to the intracellular niche of the bacteria, CD4 and CD8 T cells comprise most of the adaptive immune response. Other cell subsets, including B cells, regulatory T cells and non-classical MHC T cells also contribute to this response by mainly influencing CD4 and CD8 T cell responses (Jung *et al.*, 2002)

Since the 1960s when Mackaness showed that transfer of cells from *L. monocytogenes*-immunized mice, but not serum, provided protection against *L. monocytogenes* infection of naïve mice, it has been known that antibodies do not have a major role in *L. monocytogenes* infection. Since the majority of the bacteria remain intracellular during infection and spread intercellularly without encountering the extracellular milieu, *L. monocytogenes*-specific antibodies would be of limited to no use in controlling bacterial spread. However, under certain experimental conditions antibodies can affect the course of infection. Although infection itself does not generate high titers of antibodies, a monoclonal antibody against the pore-forming virulence factor LLO can provide protection by acting intracellularly to neutralize LLO and block bacterial escape from the phagosome (Edelson and Unanue, 2001).

2.16 Clinical Diagnosis of Listeric Infections

Listeria is found in the environment and most people are exposed to it regularly. Therefore, there is no clinical value in performing laboratory testing on asymptomatic patients, even if they are at higher risk.

For symptomatic patients, diagnosis is confirmed only after isolation of *Listeria monocytogenes* from a normally sterile site, such as blood, spinal fluid (in the setting of nervous system involvement), or amniotic fluid/placenta (in the setting of pregnancy). Stool samples are of limited use and are not recommended. *Listeria monocytogenes* can be isolated readily on routine media, but care must be taken to distinguish this organism from

other Gram-positive rods, particularly diphtheroids. Selective enrichment media improve rates of isolation from contaminated specimens (Lovett *et al*., 1987). Cultures will take 1-2 days for growth. Importantly, a negative culture does not rule out infection in the presence of strong clinical suspicion. Serological tests are unreliable, and not recommended at the present time (CDC, 2013).

Diagnosis of *Listeria* traditionally involves culture methods based on selective pre-enrichment and plating. This is followed by the characterization of *Listeria* spp using colony morphology, sugar fermentation, haemolytic properties and molecular methods (Gasanov *et al.,* 2005; Paoli *et al.,* 2005). Rapid tests have also been developed for identification of the different *Listeria* species. Conventional microbiological methods are usually very sensitive and remain the "Gold standard" (Jantzen *et al*., 2006).

Current standard methods for isolation includes

(i) Enrichment and plating methods: According to most regulatory agencies, isolation methods must be capable enough to detect one *Listeria* organism per 25g of food. This sensitivity can only be achieved by using enrichment methods. These methods employ antimicrobial agents that specifically suppress competing microflora, prior to plating onto selective agars and confirmation of cultures. The selective agents commonly used in enrichment broths are acriflavin, which inhibits the growth of other Gram positive bacteria; nalidixix acid, which inhibits Gram negative bacteria; and cycloheximide, which inhibits fungi. Other antimicrobials often used include the broad spectrum agents ceftazidime and moxalactam as well as lithium chloride. Another important characteristic of *Listeria*

isolation media is the inclusion esculin. All *Listeria* spp. hydrolyse esculin and the inclusion of esculin and ferric iron in enrichment or plating media results in the formation of an intense black color (Fraser and Sparber, 1988).

Two of the most widely-used culture reference methods for detection of *Listeria* in all foods are the FDA bacteriological and analytical method (BAM) (Hitchins, 2001) and the International Organization of Standards (ISO) 11290 method. Both of these methods require enrichment of a 25 g food sample in a selective broth, designed to slow the growth of competing organisms, prior to plating onto selective agar and biochemical identification of typical colonies.

For the FDA BAM the sample is enriched for 48 h at 30°C in *Listeria* Enrichment broth (LEB, FDA BAM formulation) containing the selective agents acriflavin, naladixic acid and the antifungal agent cycloheximide. Enriched broth is then plated onto selective agar (Ox-ford, PALCAM, MOX or LPM).

The ISO 11290 Method has a two-stage enrichment process: the food sample is first enriched in half Fraser broth for 24h, then an aliquot is transferred to full strength Fraser broth for further enrichment. Fraser broth also contains the selective agents acriflavin and naladixic acid as well as esculin, which allows detection of ß-D-glucosidase activity by *Listeria*, causing a blackening of the medium. Both the primary and secondary enriched broth are plated on Oxford and PALCAM agars.

Enrichment methods, which usually take about 30–48 h, are followed by the identification of the enriched microorganisms. Most selective agars for isolation and identification of

Listeriae (Oxford, PALCAM, MOX). rely on the esculinase reaction based on β-D-glucosidase activity to differentiate *Listeria* from other bacteria.

Typical *Listeria* colonies appear black with a black zone in surrounding medium. These media also contain selective agents to inhibit the growth of most other organisms. However, some other organisms will grow on selective plates and some species, such as *Enterococcus* and *Bacillus* spp., also utilize esculin and may have a similar appearance. Further tests are required to identify *Listeria* colonies conclusively, but suspect colonies on selective agar must first be investigated for purity. Traditionally, suspect cultures were plated onto tryptose soy agar and colonies were examined by oblique lighting, a technique in which *Listeria* colonies appear reticulated with a distinct blue-green cast (Welshimer, 1981). Currently, suspect bacteria are usually classified as *Listeria* if they display the following characteristics: Gram-positive rods, aerobic and facultatively anaerobic, non-spore forming, catalase-positive (although there are reports of catalase negative *Listeria),* oxidase-negative, fermentative in sugars and producing acid without gas. Most strains are motile at 28°C and non-motile at 37°C (Welshimer, 1981). Commercial identification kits are a widely-used alternative to traditional biochemical testing, which is time consuming and takes a week for differentiation of species using sugar utilization tests. Test strips such as API *Listeria* (bio-Merieux, Marcy-Etoile, France) and Micro-ID™ (Remel, USA) have been extensively validated and are now incorporated into standard methodology (Hitchins, 2001).

(ii) Differentiation of all the species using biochemical tests: this is based on fermentation of different sugars producing acid without gas and the Christie, Atkins, Munch-Petersen (CAMP) tests which allows differentiation of the species of *Listeria.*

The CAMP test (Groves and Welshimer, 1977) can be used to differentiate between haemolytic *Listeria* species; *L. monocytogenes, L. ivanovii and L. seeligeri.* This test is carried out by streaking a ß-hemolysin-producing *Staphylococcus aureus* strain and *Rhodococcus equi* parallel to each other on a blood agar plate. Suspect cultures are streaked at right angles in between (but not touching) the two streaks. Hemolysis by *L. monocytogenes* and to a lesser degree *L. seeligeri* is enhanced in the vicinity of *S. aureus* and hemolysis by *L. ivanovii* is enhanced in the vicinity of the *R. equi* streak. However, the CAMP test was examined for its applicability and it was shown that sometimes it cannot correctly differentiate between *L. monocytogenes* and *L. ivanovii* (Vazquez-Boland *et al.*, 1990). Therefore, a variation of the original CAMP test using commercially available ß-lysin discs is recommended in the USDA method (USDA, 2002). The identification of non-haemolytic species is based on differences in fermenting certain sugars.Whilst these biochemical identification markers are useful, they are sometimes difficult to interpret because the colour reactions can be ambiguous.

Table 2.2: Phenotypic identification of Listeriae

		L.monocytogenes	*L.innocua*	*l..ivanovii*	*L.seeligeri*	*L.welshimeri*	*L.grayi*
Hemolysis		+	-	+	+	-	-
Catalase		+	+	+	+	+	+
Oxidase		-	-	-	-	-	-
Sugar Fermentation	L-Rhamnose	+	+/-	-	-	+/-	+/-
	D-mannitol	-	-	-	-	-	+
	D-Xylose	-	-	+	+	+	-
	α -Methyl-Mannoside	+	+	-	-	+	+

Source: Gasanov *et al*., 2005

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(iii) Molecular tests: Identification of *Listeria* spp. and *L. monocytogenes* using molecular methods such as Polymerase Chain Reaction (PCR), e.t.c, is becoming increasingly popular because these techniques are extremely accurate, sensitive and specific (Gasanov *et al.*, 2005).

The PCR is now established as a reliable and reproducible technique for identification of *Listeria* spp. and, more importantly, for the differentiation of *L. monocytogenes* from other *Listeria* species using primers targeting genes of virulence factors or RNA sub-unit genes. Multiplex PCR, where multiple primer sets are used, allows the simultaneous detection of more than one pathogen in the same sample, such as *L. monocytogenes* and other *Listeria* species (Wesley *et al*., 2002). Another adaptation of PCR that also uses multiple primer sets is nested PCR; however, these primer sets are used in sequential reactions and are designed against the same target. It is used to increase sensitivity and specificity and has been used for the identification of *L. monocytogenes* in clinical samples, environmental samples and milk samples (Ha *et al*., 2002).

(iv) Rapid tests: It can take up to five days to obtain a result using traditional methods of detection for *Listeria* spp. For this reason a substantial number of alternative rapid screening methods have been developed to produce results more quickly for food and environmental samples. Many of these are available commercially and have been successfully validated by the AOAC and/or AFNOR (AOAC, 1995). The AOAC database of performance tested methods contains almost 50 products for the rapid detection of *Listeria* spp. and *L. monocytogenes* – more than for any other foodborne pathogen.

Listeria spp. and *L. monocytogenes* rapid test and screening kits utilise several different technologies, including immunomagnetic separation, EIA- and ELISA-based assays incorporating fluorescent or colorimetric detection, simple lateral flow assays incorporating immunochromatographic technology, and molecular techniques such as DNA hybridisation and PCR-based assays, many of which now include real-time detection.

Many rapid confirmation and identification methods have been developed for *Listeria* spp. and a large number have been developed into commercial products. In addition many of the rapid detection kits can also be used for confirmatory testing. Example of some of the rapid tests include API Listeria, Microgen^{TM} Listeria-ID, Microbact^{TM} Listeria 12L e.t.c.(Rapid Microbiology, 2015).

2.17 Treatment for Listeriosis

Listeriosis is treated with antibiotics. A person in a higher-risk category (pregnant women, older adults, and people with weakened immune systems) who experience fever and other non-specific symptoms, such as fatigue and aches, within 2 months of eating contaminated food should seek medical care and tell the physician or health care provider about eating the contaminated food (CDC, 2011).

If a person has eaten food contaminated with *Listeria* and does not have any symptoms, most experts believe that no tests or treatment are needed, even for persons at higher risk for listeriosis.

In non-invasive listeriosis, the bacteria will often remain within the digestive tract, causing mild symptoms lasting only a few days and requiring only supportive care. Muscle pain and fever in mild cases can be treated with over-the-counter pain relievers, and diarrhoea and gastroenteritis can be treated with over-the-counter medications if needed (CDC, 2011)

In invasive listeriosis, the bacteria has spread to the bloodstream and central nervous system. Treatment includes intravenous delivery of high-dose antimicrobials and in-patient hospital care. Duration of hospital care will vary depending on how widespread the infection is, but is usually not less than 2 weeks. Ampicillin, penicillin, or amoxicillin are often given for invasive listeriosis, and gentamicin is often added in patients with compromised immune systems. Trimethoprim-sulfamethoxazole, vancomycin, and fluoroquinolones can be used in cases of allergy to penicillin. For treatment to be effective, the antibiotic must penetrate the host cell and bind to penicillin-binding protein-3 (PBP3). Cephalosporins are not effective for treatment of listeriosis (Temple *et al.,* 2000)

Prompt treatment of *Listeria* infections in pregnancy is critical to prevent the bacteria from infecting the feotus, and antibiotics may be given to pregnant women even in non-invasive listeriosis. These oral therapies in less severe cases can include amoxicillin or erythromycin. In addition to antibiotic therapy, it often recommended that infected pregnant women receive ultrasounds to monitor the health of the feotus. Higher doses of antibiotics are sometimes given to pregnant women to ensure penetration of the umbilical cord and placenta (Janakiraman, 2008).

Asymptomatic patients who have been exposed to *Listeria* are not recommended for treatment. It is recommended that these patients be informed of the signs and symptoms of the disease and to return for medical care if symptoms present (CDC, 2011).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Study Area

The study was conducted in Zaria town and its environs. Zaria is one of the major cities in Kaduna state in the north central geo-political zone of Nigeria. Zaria is located within the Northern guinea savannah zone on a plateau at a height of 2,200 feet above sea level. It lies between latitude 7^0 and 11^0 north and longitude 7^0 44´ East. Zaria is characterised by cold humid wet season and cold or hot dry seasons. Average rainfall in Zaria ranges from 1000mm to 1250mm and the average temperature ranges from 17^0C to 33^0C (Zaria, 2010).

3.2 Study Design

A cross-sectional study and a convenience sampling of clinically healthy lactating cows for raw milk from the study area was done. Yoghurt samples were purchased directly from manufacturers as well as retailers in the yoghurt business. Both samples were collected on a weekly basis for a period of four months (August to December, 2013).

3.3 Sample Size

The sample size was determined according to Thrusfield(1997).

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

Where n= sample size

d=desired absolute precision of 5% (0.05)

P_{exp}= expected prevalence (5.3% for raw milk, Morobe *et.al*.,2009; for yoghurt, 5.3% prevalence was assumed)

Therefore: $n=\frac{1.96^2 x 0.053(1-0.053)}{0.05^2}$

n=77.14

However, a total of three hundred and sixteen samples were collected (this include an additional 5% allowable error) which comes to a subtotal of 158 samples each for raw milk and yoghurt respectively.

3.4 Sample Collection/Transportation

Thirty (30) millilitre of raw milk samples were obtained from apparently healthy lactating cows in Fulani herds located in Zaria, Kaduna State. Raw milk was collected from teats of lactating cow into sterile sample bottles. The age of each cow was recorded as well as the pH of milk was taken with the aid of a digital pH meter, whilst the age was ascertained based on the dentition of each cow.

Dairy product (Yoghurt) samples were purchased in their original package from different points of sale (Samaru, Shika, Sabo, PZ, A.B.U., Kongo, Palladan, Hanwa and Kwangilla) based on their availability in Zaria, Kaduna State.

All samples were aseptically collected and transported to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, where they were enriched immediately. However, raw

milk samples were stored in the refrigerator at a temperature of $4^{o}C$ for not more than 24hours before pH were recorded.

3.5 Materials Used.

3.5.1 Bacteriological culture media

All media used except otherwise stated were products of Oxoid® Ltd, Basingstoke, UK. The materials used include Brain Heart infusion broth, *Listeria* selective enrichment broth 500g , *Listeria* selective enrichment supplement(SRO 141E), *Listeria* selective agar 500g, *Listeria* selective supplement (SRO 227E), Nutrient agar base 500g, Bile Aesculin agar 500g, Blood agar base 500g, Tryptone soya agar 500g, Mueller Hinton agar, 500g.

3.5.2 Sugars

Rhamnose, Mannitol, Mannose, Inositol, Sucrose.

3.5.3 Antibiotic impregnated disks and their concentrations

Sulphamethoxazole - Trimethoprim (SXT, 25µg) Gentamicin (CN, 10µg), Amoxicillin/ Clavulanic acid (AMC, 30µg), Chloramphenicol (C, 30µg), Nitrofurantoin (F,300µg), Kanamycin (K, 30µg), Ampicillin (AMP, 10µg), Tetracycline (TE, 30µg), Erythromycin

(E, 15µg), Ciprofloxacin (CIP, 5µg), Cefixime (CFM, 5µg). All were obtained from Oxoid(R) Ltd, Basingstoke, UK.

3.5.4 Test kits

Microgen™ Listeria – ID System obtained from Microgen Bioproducts Ltd, UK.

3.5.5 Materials for molecular characterization

DNA extraction kit (ZYMO®, Inqaba biotech, South Africa), Primers (ZYMO®, Inqaba biotech, South Africa), Fermentas® Master mix, Agarose.

3.5.6 Other materials used

Disposable petri dishes, conical flasks (100ml, 250ml, 500ml), 5 and 10ml bijou bottles, test tubes, foil paper, masking tapes, permanent marker, hydrogen peroxide (3%), oxidase strip, nose mask, hand gloves, normal saline.

3.6 Isolation and Characterization of *Listeria* Species from Raw Milk and Yoghurt Samples

3.6.1 Isolation procedure for *Listeria* species

Listeria spp. was isolated according to the procedure described by Food and Drug Administration-Bacteriological Analytical Manual (FDA-BAM, 1997). Aseptically 10ml of each milk or yoghurt sample was added to 90ml of *Listeria* enrichment broth (LEB) containing selective *Listeria* enrichment supplement in a conical flask and incubated at 37^{o}C for 24hrs. A loopful of the enrichment culture was streaked onto containing Listeria selective agar (Oxoid SR 141E). Plates were incubated at 37^{o}C for 24h-48h. The culture plates were examined for grayish colonies having black halos and sunken centers (Ribeiro and Carminati, 1996) suspected to be *Listeria* spp.

3.6.2 Identification of *Listeria* species

Presumptive colonies based on colony morphology were subsequently subjected to Gram staining as described by Cheesebrough, (2000). Gram positive rods that occurred singly or in short chains were suggestive of *Listeria* spp**.**

3.6.3 Conventional biochemical tests for *Listeria* species

Conventional biochemical tests were carried out by the methods described by Barrow and Feltham (1995). Identification was done following a series of biochemical tests: catalase

test, aesculin hydrolysis, haemolysis on blood agar, oxidase test and sugar fermentation test (Rhamnose, mannitol, mannose, sucrose and inositol).

3.6.3.1 Catalase test

Suspected isolates were tested for their ability to produce catalase enzyme. A drop of 3% hydrogen peroxide was placed on a slide and a colony of the suspected isolates was picked with a sterile wire loop and suspended in the hydrogen peroxide. A positive reaction was indicated by formation of bubbles indicating the production of oxygen gas and water.

3.6.3.2 Hydrolysis of aesculin

Isolates suspected to belong to the *Listeria* species were subjected to this test. Bile Aesculin agar (Oxoid$^{(R)}$) was prepared according to manufacturer's instructions. The suspected isolates was streaked on the medium and incubated at 30^{o}C for 24 hours. Blackening of the medium indicated a positive reaction.

3.6.3.3 Haemolysis on blood agar

The *Listeria* species suspected isolates were grown on 5% sheep blood agar to obtain isolated colonies and a clear zone of clearing around the colonies upon incubation at 37^{o}C for 18-24 hours indicated ß – haemolysis, also α haemolysis with a zone of partial clearing and γ haemolysis with no zone of clearing around the differrent colonies were observed.

3.6.3.4 Oxidase test

Suspected isolates were tested for their ability to produce cytochrome c oxidases. A sterile wire loop was used to aseptically transfer a colony of the suspected isolates unto the surface of the oxidase detection strip; the colony was spread over the surface of the strip and then observed for three minutes for colour change to purple. Absence of colour change was indicative of a negative reaction and the inability of the test organism to utilize oxygen for energy production.

3.6.3.5 Sugar fermentation tests

Carbohydrate fermentation tests (1% Mannitol, 1% Rhamnose, 1% Maltose, 1% Innositol and 1% Sucrose) as described by Seelinger and Jones (1986) and Allerberger (2003) was carried out on the isolates to confirm and distinguish the various *Listeria* spp. About 10ml of 2% bromothymol blue indicator was added to 900ml of peptone water and sterilized at 115^{o}C for 20minutes. 10g of appropriate sugars was dissolved in 90ml of water and steamed for 30minutes. This was added to the sterile peptone water. Five milliliters were distributed into sterile tubes and a loop full of the suspected isolates grown on *Listeria* selective agar was inoculated into it and incubated for 24-72 hours at 37^{O}C. A change in colour to yellow was indicative of fermentation. Fermentation of rhamnose, maltose and sucrose but not mannose and inositol was considered positive for *Listeria monocytogenes.*

3.6.4 Microgen^{TM} Listeria ID-System testing for *Listeria* species.

The Microgen^{TM} Listeria ID -System a standardized micro-substrate system was used for identification of *Listeria* species. Reactions occurring during the incubation period are demonstrated by easily interpreted colour changes. Organism identification is based on pH change and substrate utilization. Once each reaction was recorded on the report form, each block of three reactions was converted into a numeric value to obtain 4 digit of the Microgen Octal code which was fed into a computer aided identification package for interpretation.

The substrates for Microgen^{TM} Listeria - ID system are aesculin, mannitol, xylose, arabitol, ribose, rhamnose, tetrahalose, tagatose, glucose-1-phospate, M-D-Glucose, M-D-Mannose and haemolysis.

3.7 Determination of the Susceptibility of *Listeria monocytogenes* to commonly used Antibiotics.

The susceptibility of *Listeria monocytogenes* isolates to 11 different antibiotics was tested by the agar disc diffusion method of Kirby-Bauer, (1966).

The isolates were spread unto Mueller Hinton agar plates and the antibiotic impregnated discs were apllied onto the the inoculated plates using disk dispenser. The plates were then incubated at 37^0C for 24hrs, after which clear zones of inhibition for each antibiotic were measured using transparent ruler; the results were interpreted according to the

recommendations of the Clinical and Laboratory Standard Institute (CLSI, 2011) using *Staphylococcus aureus* ATCC 25923 as control strain (Rodas-Suarez *et al*., 2006).

The multiple antibiotics resistance (MAR) index was determined for each of the isolates. This was obtained by dividing the number of antibiotics to which each isolate was resistant (a) by the total number of antibiotics to which the isolate was tested (b). The formula used was:

MAR index=a/b

3.8 Molecular Characterization of Isolates

3.8.1 Protocol used for DNA extraction of *Listeria monocytogenes*

Listeria monocytogenes were grown on selective media and incubated at 37^{o}C for 48hours.Three colonies of each isolate were picked, inoculated into 5ml of tryptone soya broth and incubated at 37^{o}C for 24hours. 1.5ml of the broth culture of each isolate was centrifuged at 10 000 rpm for 3minutes in a micro-centrifuge tube to obtain the bacterial cells and vortexed for 5minutes to suspend the cells. Two hundred micro litres of the suspension was added to Zymo Research bashing bead lysis tube and 750µl solution was added to the tube. This was vortexed for 5minutes. The tube and its contents were centrifuged at 10,000 rpm for 2minute. Four hundred microlitres supernatant was transferred to a Zymo-spin filter in a collection tube and centrifuged at 7000rpm for 2 minute. Subsequently, 1200µl of bacterial DNA binding buffer was added to the filtrate in the collection tube from the previous step. In the next step 800µl of the mixture was

transferred to Zymo spin 11C column in a collection tube and centrifuged at 10.000rpm for 2 minute. The flow through from the collection tube was discarded and this step repeated. Then, 200µl of DNA pre- wash buffer I was added to the Zymo spin 11C column and centrifuged at 10,000rpm for 2 minute. Five hundred microlitres bacterial DNA wash buffer II was then added to the Zymo spin 11C column and centrifuged at 10 000rpm for 2 minute. The Zymo spin 11C column was transferred to a clean 1.5ml micro centrifuge tube and 50µl of DNA elution buffer was added directly to the column matrix and centrifuged at 10 000rpm for 1 minute. DNA was collected in the micro centrifuge tube.

3.8.2 Detection of the virulence associated gene of *Listeria monocytogenes* using conventional PCR

A total of 10 DNA obtained from raw milk from this study based on conventional biochemical testing were used. DNA of the following *Listeria monocytogenes* isolates was used: SM1, SM17, SM47, SM52, SM56, SM66, SM84, SM86, SM87 and SM118.

PCR Amplification

Amplification of virulence associated gene (*hylA*) product was done by PCR using reverse and forward primers (D1-Forwad; 5'CCT AAG ACG CCA ATC GAA – 3'; D1-Reverse; 5' AAG CGC TTG CAA GTC CTC –3', to yield a 702bp product. Amplification was carried out in a mixture that consisted of: Taq DNA polymerase, Primer, distilled water, DNA template, PCR buffer, dNTPs (dATPs, dGTPs, dTTPs, dCTPs), MgCl2. Amplification was carried out in a 50µL reaction mixture that consisted of 4µl of DNA

template with 1µL of Tag polymerase, 26µL of distilled water, 4µL of $MgCl_2$, 10µL of PCR buffer, 1µL of dNTPs and 4µL of primers. PCR cycling conditions was done as follows; initial denaturation at 95^0C for 5 minutes followed by 35 cycles each of denaturation at 94^0C for 1min, annealing at 68^0C for 30sec and extension at 72^0C for 1 minute and final extension at 68^0C for 7 minutes after which the product was cooled and held at 4^0C.

3.8.3 Agarose gel electrophoresis and documentation.

A 1.5% agarose gel was prepared by suspending 1.5g agarose powder in 100mls of distilled water. This was heated for 3 minutes in a microwave oven after which it was allowed to cool to50^oC and ethidium bromide at a concentration of 0.5µg/ml was added. It was then poured into the agarose gel chamber with the comb and allowed to solidify. Ten micro litre of each PCR product was mixed with 0.2ml of loading dye which was then resolved in 1.5% agarose gel at 100V for 45 minutes loaded into the wells of the gel. A 100bp DNA molecular ladder (Finnzymes, Espoo, Finland) was also used as a molecular mass marker. A UV transilluminator (UVtec, Sigma, Germany) was used to view the bands of the PCR products and bioprofile gel documentation system (Mitsubishi, Tokyo, Japan) was used to generate a final print of the gel picture.

3.9 Data Analysis

The data obtained was analyzed using statistical package for social science (SPSS version

20.0) .Data obtained were presented using descriptive statistics such as tables and charts. Fishers exact test was used to establish association between *Listeria* spp, the age of the animal and pH of raw milk. Chi square was also used to test the association of *Listeria* spp in yoghurt and raw milk samples. Values of $P< 0.05$ were considered significant.

CHAPTER FOUR

RESULTS

4.1 Prevalence of *Listeria*

Three hundred and sixteen samples of yoghurt and raw milk from Zaria, Kaduna State, were analyzed, which yielded 36 isolates of *Listeria* species based on conventional biochemical testing giving an overall prevalence rate of 11.4%. The raw milk samples had the highest isolation rate of 20.3%, while for yoghurt the rate was 2.5 % (Table 4.1). Of the 36 isolates, 10 were identified as *Listeria monocytogenes* giving a prevalence rate of 3.2%.

The 36 isolates of *Listeria* species were further tested using Microgen^{TM} Listeria -ID system which resulted in the identificaation of 1 *Listeria monocytogenes,* 21 *L. invanovii.*, 13 *L. grayi* and 1 *L. seeligeri* respectively (Table 4.2).

The pH of the raw milk and the ages of the animals were analyzed using the Fishers exact test (FET) which gave a value of p= 0.049 (Table 4.3) for the pH. Thus there was a statisticaly significant association between the occurrence of *Listeria* and the pH of the raw milk. The age of the animals was not found to be statistically significant with respect to the occurrence of *Listeria* in milk samples (p=0.346) (Table 4.4).

Table 4.1 Prevalence of *Listeria* species from Yoghurt and Raw milk Samples from Zaria, Kaduna State, Nigeria based on conventional biochemical testing.

Sample Type	No of samples Tested	No (%) of *Listeria* spp isolated	No (%) of *L.m* i*solated
Yoghurt	158	4(2.5)	0(0.0)
Raw Milk	158	22(13.9)	10(6.3)
Total	**316**	**26(8.2)**	**10(3.2)**

Chi square value (χ^2)=1.042,* P=*0.307
****L.m= Listeria monocytogenes***

Table 4.2 Distribution of *Listeria* species in Yoghurt and Raw milk samples in Zaria, Kaduna State, Nigeria based on Microgen™ *Listeria* -ID System.

Sample Type	No. of *L.monocytogenes*	No. of *L.ivanovii*	No. of *L. grayi*	No. of *L.seeligeri*	Total
Yoghurt	0	2	2	0	4
Raw milk	1	19	11	1	32
Total	**1**	**21**	**13**	**1**	**36**

Table 4.3: Distribution of *Listeria* species based on the pH of Raw Milk Samples in Zaria, Kaduna State, Nigeria.

pH	No. (%) *L. monocytogenes*	No. (%) *Listeria* species
< 7	7(53.8%)	6(46.2%)
≥ 7	3(15.8%)	16(84.2%)

Fishers Exact Test (FET) = 0.049*

Table 4.4 Distribution of *Listeria* species based on age of cows sampled in Zaria, Kaduna State, Nigeria.

Age of animal (Years)	No of *L. monocytogenes* (%)	No. of *L.* species (%)	Total (%)
1-4	3(50)	3(50)	6(100)
5 and above	7(26.9)	19(73.1)	26(100)

FET=0.346

4.2 Antimicrobial Susceptibility of *Listeria monocytogenes* Isolates

All the 10 *L. monocytogenes* isolates from raw milk were found to be resistant to one or more of the 11 antibiotics used (Table 4.5). All the 10 isolates, were found to be susceptible to gentamicin (100%) but with various frequencies of susceptibilitities to other drugs; kanamycin (90%) ciprofloxacin (80%), chloramphenicol (70%), amoxicillin/ clavulanic acid (60%), erythromycin (50%), both sulphamethoxazole/trimethoprim, and nitrofurantoin (30%), tetracycline (20%) and cefixime (10%). However, all isolates were resistant to ampicillin.

The antibiotic susceptibility testing gave 9 antimicrobial resistance patterns (Table 4.6). The resistance patterns showed that the most common pattern was F, TE, CFM, AMP and SXT with a frequency of 20.0% (Table 4.6), while all the others occurred once. Ampicillin resistance was the most common followed by Cefixime and tetracycline among the various patterns observed.

The lowest Multiple Antibiotics Resistant (MAR) Index was 0.27 and the maximum 0.81 (Table 4.7).

4.3 Hemolysis of *Listeria monocytogenes* on Sheep Blood Agar

Listeria monocytogenes on blood agar formed colonies of about 1-2mm in diameter after 24hours of cultivation. Colonies were β-hemolytic, however isolates produced only narrow zones of hemolysis around the colonies.

4.4 PCR Detection of the Virulent Gene of *Listeria monocytogenes*.

All the isolates were negative for *hyl* A gene by PCR.

Table 4.5 Antimicrobial susceptibility of 10 *Listeria monocytogenes* isolates from Yoghurt and raw milk from Zaria, Kaduna State, Nigeria.

Antibiotic and disk conc (µg)	No. (%) Susceptible	No. (%) Resistant
Gentamicin (10)	10(100.0)	0(0.0)
Kanamycin (30)	9(90.0)	1(10.0)
Ciprofloxacin(5)	8(80.0)	2(20.0)
Chloramphenicol (30)	(70.0)	3(30.0)
Amoxycillin/Clavulanic acid (30)	6(60.0)	4(40.0)
Erythromycin(15)	5(50.0)	5(50.0)
Sulphamethoxazole/ Trimethoprim (25)	3(30.0)	7(70.0)
Nitrofurantoin (300)	3(30.0)	7(70.0)
Tetracycline (30)	2(20.0)	8(80.0)
Cefixime (5)	1(10.0)	9(90.0)
Ampicillin (10)	0 (0.0)	10 (100.0)

Table 4.6 Antimicrobial Resistance Patterns of 10 *Listeria monocytogenes* Isolates from Raw Milk in Zaria, Kaduna State, Nigeria.

S/N	Resistance Patterns*	Frequency of occurrence
1	TE, CFM, AMP	1
2	CFM, AMP, SXT	1
3	F, CFM, AMP, SXT	1
4	F, TE, CFM, AMP, SXT	2
5	E, TE, CFM, AMP, SXT	1
6	E, F, C, TE, AMP, AMC	1
7	E, F, TE, CFM, AMP, CIP, SXT, AMC	1
8	E, F, C, TE, CFM, AMP, SXT, AMC	1
9	E, F, C, TE, CFM, AMP, CIP, AMC, K	1

Antibiotics:* E, erythromycin; F, nitrofurantoin; C, chloramphenicol; TE.tetracycline; CFM, cefixime; AMP, ampicillin; CIP, ciprofloxacin; SXT, sulphamethoxazole/trimethoprim; K, kanamycin; AMC, amoxicillin/clavulanic acid

Table 4.7 Multiple Antibiotics Resistance (MAR) Index for 10 *L. monocytogenes* Isolate from Raw Milk in Zaria, Kaduna State.

Isolate	No. of Antibiotics to which to which each isolate was resistant	MAR Index*
SM1	3	0.27
SM17	3	0.27
SM47	8	0.73
SM52	6	0.55
SM56	9	0.81
SM66	8	0.73
SM84	4	0.36
SM86	5	0.45
SM87	5	0.45
SM118	5	0.45

*****Eleven (11) antimicrobial agents were tested.

CHAPTER FIVE

DISCUSSION

In many developed countries listeriosis has been detected in raw milk and dairy products and are regularly reported in Europe and North America, but in Africa and other developing countries, Nigeria inclusive, where the food industries are not very developed, only a few sporadic cases have been reported (Boukadidda *et al.,* 1994; David and Odeyemi, 2007). The results obtained from this study have however revealed that this organism is present in raw milk and dairy products such as yoghurt. Apart from detecting *Listeria* species in raw milk and yoghurt, it has also been shown that yoghurt and raw milk may be vehicles for the transmission of food borne listeriosis to humans (Adetunji and Arigbede, 2011).

Listeria monocytogenes remains a serious threat to Public Health and an appreciable economic burden to the food industry. Numerous studies have shown that dairy foods produced from contaminated products are significant sources and vehicles of transmission for food borne listeriosis (Adetunji and Arigbede, 2011).

The prevalence of *Listeria* species in yoghurt and raw milk samples was found to be 11.4%, which agrees with that reported by Aisha and Kawo (2014) from ready to eat food retailed in Kano state Nigeria, in which they reported a prevalence of 11.3%. However, this is higher than that reported by Aygun and Pehlivanlar (2006) from raw milk in Turkey in which they reported a prevalence of 2.12%. The difference in prevalence could be as a result of the different geographical locations, type of sample or possibly due to methods of isolation and identification.

The 3.2% overall prevalence of *Listeria monocytogenes* agrees with the report of Mahmoodi (2010) in Noorabad, Iran where he reported a prevalence of 3.3%. However, this prevalence is lower than the 20.0% repoted by Adetunji and Olaoye (2012) from milk samples in South West Nigeria as well as that of Boubendir *et al.* (2011) from Algeria and Morobe *et al.*(2009) in Gaborone Botswana who obtained prevalences of 5.76 and 5.3% respectively from milk samples.

No *Listeria monocytogenes* was isolated from any of the yoghurt samples tested. This may be attributed to the high acidity of the product and prior milk pasteurization and also the addition of starter cultures for fermentation which act as effective barriers to the growth of pathogens including *L. monocytogenes* (Liu and Puri, 2008). This result is consistent with previous reports by Mahmoodi (2010), Aisha and Kowa (2014), Brito *et al.* (2008) and Neves *et al.* (2008), all of whom failed to isolate *Listeria monocytogenes* from Yoghurt. According to Aygun and Pehlivanlar (2006), exposure to pH 4.0 can totally inactivate *Listeria monocytogenes* in food. However, noteworthy is the fact that *Listeria ivanovii* which is also of public health threat was isolated from yoghurt and thus maybe a vehicle for the transmission of the agent to human.

Contamination of fermented dairy products with *Listeria spp.* such as *Listeria ivanovii* has been reported in Africa (Walker *et al.*, 1990). This has been attributed to the differences in hygiene conditions during milking, handling, processing and storage of raw and fermented dairy products (Dalu and Feresu, 1990). Studies have also suggested that fermented dairy products may be contaminated with *L. monocytogenes* and other *Listeria spp.* from the environment, diseased udders, ingredients and packaging materials (Conner *et al.*, 1990).

Pasteurization as a thermal process may be used to reduce food borne pathogens including *Listeria* but studies have shown that it can survive the pasteurization conditions.

The relative occurrence (5.6%) of *Listeria ivanovii* in sampled yoghurt may be attributed to lack of effective sanitary precautions and less careful or unhygienic handling procedures during milking process and yoghurt production. The use of traditional milking methods that maybe practiced by small scale producers also expose milk to pathogenic bacteria found in cow udders and probably, on the hands of the milkers who might have come in contact with faeces of the cows. Although pasteurization kills the pathogens in milk, contamination can still occur during packaging. Improperly performed pasteurization contamination of the starter culture inocula, slow rate of acid formation and pH decline and the the occurrence of contamination post pasteurization are the most likely explanations for the presence of *Listeria ivanovii* in sampled yoghurt obtained in this study.

There was a statistically significant association ($P < 0.05$) between the pH of milk and isolation of L*isteria* spp. A number of studies have demonstrated that *L. monocytogenes* is more acid tolerant than most food-borne pathogens, although the sensitivity of the organism to organic acids varies with the nature of the acidulant used (Clive and Park, 2001). *Listeria* spp. prefers to grow at pH 7-8 but they will grow in the range pH 5-10 and may survive and grow in material with a pH as low as 4.4. The type of acid and the storage temperature have a marked effect on the ability of *Listeria* to survive and grow at low pH (Clive and Park, 2001). *L. monocytogenes* has been reported to possess variable heat resistance depending on conditions of medium such as pH, acidulant, fatty acid composition, storage temperature and lysozyme content (Ribeiro and Carminatti, 1990). Plastic is rather difficult to clean,

absorbs extraneous flavors and is conducive for the formation of biofilms. *L. monocytogenes* has been reported to adhere to polymeric materials, including plastic and stainless steel, especially at temperatures greater than 30^0C and low pH (4 to 7) (Mosteller and Bishop, 1993).

From this study, data obtained has shown that as acidity increases the activity of *Listeria* species are inactivated, but with an increased alkalinity (high pH) there is a preponderance of *Listeria* organisms (Clive and Park, 2001). This however, is not conclusive for *L. monocytogenes* as the data has shown that even at low pH (Mosteller and Bishop, 1993), there is still likelihood of the presence of this pathogen.

There was no statistical significance ($P>0.05$) between the occurrence of *Listeria* spp with the ages of the animal. This is true because the intestines of animals (Lorber, 1996) regardless of the age have been found to harbour *Listeria* organisms, hence serve as carrier in the shedding and transmission of *Listeria* organisms to the environment and man. These organisms are known environmental contaminants and different age groups of animals are exposed to the environment at the same time.

Antimicrobial susceptibility of 10 *Listeria monocytogenes* isolates showed that all the isolates were susceptible to the aminoglycoside gentamicin, which agrees with the works of Ennaji *et al.* (2008), Ndahi, (2011) and Aureli *et al.* (2003) who respectively reported a 100% susceptibility.

The present study showed that 100% of the isolates were resistant to the antimicrobial agent ampicillin; this report is at par with those obtained by Ndahi, (2011) and Yakubu *et al.*

(2012b) who individually reported 100% resistance to ampicillin. However, this is completely different from the findings of Ennaji *et al.* (2008) and Aureli (2003), who respectively reported that ampicillin showed good activity (100%) against most *L.monocytogenes* isolates from meat samples.

The resistance (70.0%) shown by the Folate pathway inhibitor to sulphamethoxazole/ trimethoprim is similar to the work of Ndahi (2011) who reported 100% resistance to this antibiotic, but differs with those of Ennaji *et al.* (2008) and Yucel *et al.* (2005) who independently found a high sensitivity(90% and 66% respectively) by their isolates to the antibiotic. The third generation cephalosporin Cefixime showed very poor activity (10%) to the organisms, in agreement with the works of Ennaji *et al.* (2008).

The present study showed a very high percentage resistance (90%) by the isolates to tetracycline which is similar to the findings of Ndahi (2011) and Ieren *et al*. (2014) who independently reported 100.0% resistance of isolates from meat samples and (71.4%) in processed and unprocessed vegetable salad.

The high rate of resistance of *Listeria monocytogenes* to tetracycline obtained in this study may be due to the fact that it is the most commonly available antibiotic used as growth promoter and routine chemoprophylaxis among livestock (Olatoye, 2010) in Nigeria. This is however of significance considering that tetracycline is a first line drug in Nigeria and in most developing countries, people with gastrointestinal infections readily purchases it across the counter for self medication (Chigor *et al.*, 2010).

High frequency of resistance to ampicillin in this study which agrees with that of Ndahi (2011), may be attributable to the low cost of this drug thus making it readily available in

treating many diseases of the illiterates and it is extensively used in developing countries (Okeke *et al.*,1995).

Muitiple antibiotics resistance index of the *L.monocytogenes* isolates showed that the minimum MAR was 0.27. According to Krumpermann (1983), an MAR index above 0.2 indicates that the isolates are from a high source of contamination where antibiotics are constantly used and abused. This reflects the findings in this study where the minimum MAR index was above 0.2, a finding that is at par with the findings of Adetunji and Olaoye (2012) and Ieren *et al.*, (2013).

All isolates in this study displayed multidrug resistance to the antibiotics tested. This result is in agreement with the findings by other researchers who reported multidrug resistance among *Listeria monocytogenes* (Yakubu *et al.*, 2012a; Ennaji *et al.*, 2008). Various isolates were simultaneously resistant to 3 or more of the antibiotics tested.

Multiple antimicrobial resistance in *Listeria monocytogenes* is reported to be mediated by self transferable plasmids (Charpentier and Courvalin, 1999). According to Levin *et al.* (1997), multiple resistance capable of regional dissemination can emerge as a result of antimicrobial selection pressure in either livestock or humans.. Evidence has been found which indicate that resistant strains of pathogens can be transmitted to humans through food (Khachatourians, 1998).

The high frequency of multidrug resistance among *Listeria monocytogenes* isolates seen in this study is quite alarming. The selection and spread of resistant organisms in developing countries, which can often be traced to complex socioeconomic and behavioural antecedents, has contributed to the escalating problem of antibiotic resistance worldwide

(Okeke *et al.*, 1995)

Results from this *in-vitro* study indicates that the aminoglycosides; gentamicin, kanamycin,and the flouroquinolone, ciprofloxacin may be the drugs of choice in treating listeriosis since ≥ 80% of the isolates were sensitive to these agents. This is in agreement with the findings of Ndahi, (2011), and Ieren *et al.*, (2013).

The development of antimicrobial resistance by the *Listeria monocytogenes* isolates to these drugs may pose a major challenge in both human and veterinary medicine, because these drugs are commonly used in the treatment of human patients and in veterinary practice; and shedding of the resistant bacteria into the environment by cattle may lead to a widespread dissemination of antibiotic resistance genes to the resident bacteria in the environment (Callaway *et al.*, 2003).

The 10 isolates of *Listeria monocytogenes* from raw milk samples were all negative for the *hylA* gene using PCR. Isolates may have lost their virulence determinants as a result of spontaneous mutations (Cooray *et al.*, 1994) that may have occurred during storage on slant.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The present study evaluated the prevalence and antibiograms of *Listeria monocytogenes* in yoghurt and raw milk samples in Zaria, Kaduna State.

A total of three hundred and sixteen samples of yoghurt and raw milk from different locations in Zaria were examined for *Listeria* species. Thirty six isolates of *Listeria* species were identified using the conventional biochemical tests of which ten were *Listeria monocytogenes*, which gives an overall prevalence of 3.2%. Twenty six other isolates of *Listeria* species were also identified and gave an overall prevalence of 8.2%. *Listeria monocytogenes* was not isolated from yoghurt which may have been due to the high acidity (low pH) of yoghurt which does not favour the growth of these organisms. However, the pathogenic species also incriminated in human listeriosis, *Listeria ivanovii* was isolated from the yoghurt samples.

All the thirty six isolates were further examined using the Microgen^{TM} *Listeria* ID System. This test identified only 1 *Listeria monocytogenes*, 13 *Listeria grayi*, 21 *Listeria ivanovii* and 1 *Listera seeligeri*. In this regard the use of Microgen^{TM} *Listeria* ID System for rapid identification of *Listeria* species needs re-evaluation since it was only able to correctly identify only one of the *L. monocytogenes* isolates.

The ten *Listeria monocytogenes* isolates were subjected to antibiotic susceptibility testing using the disc diffusion technique. Eleven antibiotics used in both veterinary and human practices were included in this study. The ten *Listeria monocytogenes* isolates were all found to be susceptible to gentamicin and resistant to ampicillin. The antibiotics of choice in the treatment of listeriosis may be gentamicin, kanamycin, and ciprofloxacin based on the *in vitro* results.

The antibiotic susceptibility profiles showed multidrug resistance with 9 different resistance patterns observed. Ampicillin was the predominant antibiotic observed in the resistance patterns, followed by cefixime and tetracycline.

The pH of the raw milk was statistically significantly ($p=0.049$) associated with the occurrence of *Listeria* spp. whereas the age of the animal was not ($p=0.346$).

The findings in this study point to the possible role of yoghurt and raw milk as vehicles for food borne diseases and the transmission of multiple antibiotic resistant strains to the populace, thus their consumption has potential health risk to consumers not just in Zaria but possibly also to the nation at large.

In conclusion, the results obtained from this study showed that yoghurt and raw milk available to consumers in Zaria, Kaduna State, may be contaminated with pathogenic *Listeria* such as *Listeria monocytogenes* and *Listeria ivanovii* which may lead to human infection as have been reported in other countries. Furthermore, the detection of *Listeria*

monocytogenes in raw milk suggests that consumption of raw milk has potential health risk to consumers.

The antibiograms of *Listeria monocytogenes* isolates showed a high prevalence of resistance to ten out of the eleven antibiotics tested. The data suggests that selection pressure imposed by the use of these antibiotics whether therapeutically in human and veterinary medicine or as prophylaxis in animal production, maybe a key driving force in the selection of antimicrobial resistance by *Listeria monocytogenes.* Thus raw milk can serve as vehicle for transmission of multiple drug resistant *Listeria monocytogenes*.

6.2 Recommendations

The following recommendations are made based on the findings of the present study;

1. The practice of raw milk consumption by nomadic Fulanis, farmers, and others should be discouraged because it exposes them to possible infection with *Listeria* spp.
2. High level of hygiene should be observed during milking and production of yoghurt so as to minimize contamination between raw unprocessed milk and yoghurt.
3. There is a need to legislate and enforce laws limiting the prescription and dispensing of antibiotics to only Veterinarians. Extension workers should educate farmers, health workers, animal health practioners and the general public on the dangers of the indiscriminate use of antimicrobial agents.
4. The aminoglycosides and the flouroquinolone should be preserved by limiting their use in the treatment of listeriosis and other infections.Further studies should be done to determine the virulence genes in *Listeria monocytogenes* isolates from raw milk.

.6. Further studies should focus on identifying the specific sources of *Listeria ivanovii* in yoghurt samples as it maybe an important step in risk assessment and controlling the incidence of these organisms in raw milk and fermented dairy products.

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APPENDICES

Appendix I: Table of substrate and reactions of Microgen™ L*isteria* - ID System

		Reaction	Positive	Negative
1	Aesculine	Aesculine hydrolysis	Black	Straw colour
2	Mannitol	Fermentation of specific sugars producing acid end products changes the Bromocresol Purple indicator from purple to yellow	Yellow	Purple
3	Xylose			
4	Arabitol			
5	Ribose			
6	Rhamnose			
7	Trehalose			
8	Tagatose			
9	Glucose-1-Phosphate			
10	Methyl-D-Glucose			
11	Methyl-D-Mannose			
12	Haemolysin	Haemolysin of sheep red blood cells	Straw – Brown coloured homogenous liquid, no carpet of red cells on the well floor	Carpet of red cells on well floor. Cells may appear red – brown in colour

Well number 12 is empty and is used for an in-well haemolysis reaction when haemolysin reagent is added to bacterial suspension.

Appendix II: Chart showing the expected result on Microgen™ *Listeria* ID- System after 18-24hours

	ESC	MAN	XYL	ARL	RIB	RHA	TRE	TAG	GIP	MDG	MDM	HEM
L. monocytogenes (ATCC35152)	+	-	-	+	-	+	+	-	-	+	+	+
L .inoccua (ATCC33090)	+	-	-	+	-	+	+	-	-	+	+	-
L. welshimeri	+	-	+	+	-	+	+	+	-	+	+	-
L. seeligeri	+	-	+	+	-	-	+	-	-	+	-	+
L.ivanovii	+	-	+	+	-	-	+	-	+	+	-	+
L. grayi(ATCC19120)	+	+		+	+	-	+	-	-	-	+	-

AppendixIII: Antimicrobial inhibition zone interpretation chart.

Antimicrobial agent	Symbol	Disk content(µg)	Sensitive (mm)	Resistance (mm)
Ampicillin	AMP	10	20	19
Amoxicillin/Clavullanic acid	AMC	30	29	28
Chloramphenicol	C	30	18	16
Ciprofloxacin	CIP	5	21	15
Cefixim	CFM	5	20	16
Erythromycin	E	15	23	13
Gentamycin	CN	10	15	12
Kanamycin	K	30	18	13
Nitrofurantoin	F	300	17	14
Tetracycline	TE	30	19	14
Sulfamethoxazole-Trimethoprim	SXT	25	17	12

Adopted from Clinical and Laboratory Standard Institute (CLSI, 2011) using *Staphylococcus aureus* (ATCC 25923) as control strain.