

**OCCURRENCE OF *LISTERIA MONOCYTOGENES* IN FRESH MEAT AND
OFFAL SOLD FOR HUMAN CONSUMPTION IN ZARIA METROPOLIS,
NIGERIA**

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FOR HUMAN CONSUMPTION IN ZARIA METROPOLIS, NIGERIA**

BY

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**A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE
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**DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE
MEDICINE,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA**

JULY, 2016.

DECLARATION

I declare that the work in this DISSERTATION entitled “**OCCURENCE OF *Listeria monocytogenes* IN FRESH MEAT AND OFFAL SOLD FOR HUMAN CONSUMPTION IN ZARIA METROPOLIS, NIGERIA**”, has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine under the supervision of Prof. J. K. P. Kwaga and Dr B. V. Maikai. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project report was previously presented for another degree or diploma at this or any institution.

Shola Elijah OLANIYAN

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Signature

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CERTIFICATION

This DISSERTATION entitled “OCCURENCE OF *Listeria monocytogenes* IN FRESH MEAT AND OFFAL SOLD FOR HUMAN CONSUMPTION IN ZARIA METROPOLIS, NIGERIA” by Shola Elijah OLANIYAN meets the regulation governing the award of Masters of Science in the Department of Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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This project work is dedicated to the Lord God Almighty, my parents; Gabriel and Mary Olaniyan, siblings (Olayinka and Wilson) and all my friends. May God continue to bless and keep you all.

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ABSTRACT

Listeriosis is a disease caused by a gram positive, non-spore forming intracellular pathogenic bacteria of the genus *Listeria*, commonly associated with food. To determine the presence of *Listeria monocytogenes* and some of its associated virulence genes in fresh meat samples sold for human consumption, 240 samples were obtained comprising of 156 beef, 26 liver, 25 reproductive tract and 33 intestinal parts sold at various locations in Zaria from July to November, 2013. Sixty six (27.5%) isolates of *Listeria* species were obtained and characterised through conventional biochemical tests, out of which, 8 (3.3%) were *Listeria monocytogenes*, but only one was subsequently confirmed using the Microgen^R 12L ID kit. Beef had the highest occurrence of 66.7% followed by reproductive tract (13.6%), intestines (10.6%) and liver (9.1%). Antibiotic susceptibility testing of the eight isolates by Kirby-Bauer disk diffusion method revealed that 100% of the *L. monocytogenes* isolates were resistant to ampicillin, 87.5% to tetracycline and cefixime, 75% to amoxicillin/clavulanic acid, 62.5% to erythromycin, 50% to chloramphenicol, nitrofurantoin and sulfamethoxazole, 37.5% to ciprofloxacin and kanamycin and 12.5% to gentamicin,. The least resistance frequency was to gentamicin 12.5%. However, all the isolates were resistant to at least 3 antimicrobial agents. Antibiotic resistance profiles of the isolates revealed 6 different antimicrobial resistance patterns. Isolates were subjected to a multiplex PCR to detect the presence of the haemolysin (*hylA*), internalin (*inlA*) and invasiveness associated protein (*iap*) virulence genes. None (0%) of the eight isolates was found to harbour haemolysin *hylA*, 1(12.5%) was positive for both internalin *inlA* and invasiveness associated proteins *iap* while 5(62.5%) harboured *iap* gene. The findings of this study showed the occurrence of *Listeria monocytogenes* in fresh meat samples in Zaria

metropolis which displayed multiple antibiotic resistance and possessed virulence genes *inlA*, and *iap*. This finding is of public health significance.

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CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Listeriosis is a disease caused by a gram positive, non-spore forming intracellular pathogenic bacteria of the genus *Listeria*, commonly associated with food. *Listeria* is widely distributed in nature. The bacteria are ubiquitous saprophytes that live in plant-soil environments and have been isolated from about 42 species of domestic and wild mammals and 22 species of birds, as well as fish, crustaceans, insects, sewage, water, feedstuffs, milk, cheese, meconium, faeces and soil (Kirkan *et al.*, 2006). Although several species are recognised (*Listeria monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. marthii*, *L. rocourthiae*, *L. grayii*, *L. fleisehmanni*, *L. coloradonensis*, *L. decoloradonensis*, *L. aquatic sp. nov.*, *L. floridinensis nov.*, *L. cornellensis sp. nov.*, *L. gradensis sp. nov.*, *L. riparia sp. nov.*, and *L. murrayi*), only two, *Listeria monocytogenes* and *L. ivanovii*, are pathogenic; the former is responsible for disease in both humans and animals, while the latter causes disease mostly in ruminants but also in other animals. The other *Listeria* species are generally considered to be non-pathogenic (Low and Donachie, 1997; Brugere-Picoux, 2008). There are reports however of *L. seeligeri* and *L. ivanovii* causing illness in humans (Cummins *et al.*, 1994; Cocolin *et al.*, 2002), and *L. innocua* occasionally associated with encephalitis in ruminants (Walker *et al.*, 1994)

Individuals who are particularly susceptible to this condition are those who are immune-compromised (as in HIV/AIDS infection), pregnant women, new-born babies, and the elderly (Farber and Peterkin, 1991; McLauchlin *et al.*, 2004). Only *Listeria*

monocytogenes and *Listeria ivanovii* were found pathogenic in test animals causing death within five days. *Listeria monocytogenes* induced pathological changes characterised by abscesses in the liver, pericarditis and frank haemorrhage in the kidneys. The most notable change was the accumulation of mononuclear cells in the loose connective tissue surrounding the bile ducts.

The generic name was given in honour of a British Surgeon, Sir Joseph Lister. Pirie changed the name of the bacterium to *L. monocytogenes* from *Bacterium monocytogenes* or *Listerella hepatolytica* in 1940, and in 1948 the organism was recognised by the same name in the 6th edition of Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1948). *L. monocytogenes* was first isolated from sheep in 1929 and the first human case was reported in the same year by Nyfeldt (1929). By 1961 *L. monocytogenes* was the only known species in the genus *Listeria*.

Listeria survive wide ranges of temperature (-7°C to -45°C), pH (4.3-9.6) (Buchanan *et al.*, 1993), salt concentrations (up to 10%) (Robert and Wiedmann, 2003), grow in gas or vacuum-packaged products at refrigeration temperatures (Duffy *et al.*, 1994); and survive low water activity (Nolan *et al.*, 1992). The ability to survive and multiply under conditions frequently used for food preservation makes the bacteria particularly problematic to the food industry (Roberts and Wiedmann, 2003).

All *Listeria* species are psychotrophic and widely spread in the environment. *L. monocytogenes* has indeed emerged as a foodborne pathogen and a contaminant of various foodstuffs such as milk and dairy products, meat and meat products, vegetables and seafood.

1.2

Statement of Research Problem

It has been shown by various studies that listeriosis is a food-mediated illness (Embarek, 1994; Slutsker and Schuchat, 1999). A wide range of foods such as salads, sea-foods, meat, and dairy have been implicated in listeriosis, which follows the oral ingestion of the contaminated food (Finlay, 2001). *L. monocytogenes* has only recently been recognised as causing foodborne disease with major public health consequences. The pathogen gained prominence in the 1980s after several large outbreaks in the United States, Canada, and Europe established conclusively that contaminated foods are the primary means by which this microorganism affects human populations. The Centers for Disease Control and Prevention (CDC's) baseline estimate is that foods contaminated with *L. monocytogenes* caused approximately 2,500 cases of illness, including approximately 500 fatalities, in the United States each year. At the recommendation of CDC, the risk assessment used 2,078 cases of listeriosis and 390 fatalities as the baseline values for the national annual public health impact of the disease (CDC).

This pathogen has been implicated in major outbreaks and sporadic cases in different countries. The annual endemic disease rate varied from 2 to 15 cases per million populations, with published rates varying from 1.6 to a high rate of 14.7 in France for 1986. Zambia had 85 reported cases of meningitis due to *Listeria*. In Ethiopia, an incidence of 18.3% was recorded in raw meat (Firehiwot, 2007). In 2000, the CDC reported that of all the foodborne pathogens tracked by them, *L. monocytogenes* had the second highest case fatality rate (21%) and the highest hospitalisation rate (90.5%) after *E. coli* (El-Shamy *et al.*, 1993). In Northern Nigeria, 27% mortality rate due to *L.*

monocytogenes (serovar 4) was reported (Onyemelukwe *et al.*, 1983). There are other products, traditionally considered of low risk, which have recently been linked to listeriosis transmission, such as the large listeriosis outbreak reported in Italy due to the consumption of corn. Though no fatalities occurred, more than 1500 people were affected (Aguado *et al.*, 2004). Outbreaks have been reported in cattle in Nigeria (Akpavie and Ikheloa., 1992) and Oni *et al* (1989) isolated *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 3a, and 4b from eight species of domestic animals including horses (68.4%), pigs (36.1%), cattle (20.8%), goats (20%), dogs (20%), free range chickens (6.8%), sheep from Zaria (14.7%) and submissions to Ahmadu Bello University Veterinary Teaching Hospital (13%).

The manifestations of listeriosis include septicaemia, meningitis, encephalitis and intrauterine or cervical infections in pregnant women which may result in spontaneous abortions during the 2nd or 3rd trimester or still birth. The onset of the aforementioned disorders is usually preceded by influenza-like symptoms including persistent fever followed by nausea, vomiting and diarrhoea, particularly in patients who use antacid (Tominaga *et al.*, 2006). Once the bacterium enters the host's monocytes, macrophages or polymorphonuclear leukocytes, it is blood-borne and can grow in body cells (Sebelius *et al.*, 1999).

L. monocytogenes also produces an exotoxin *Listeriolysin* (LLO) which is a key agent in human neutrophil activation. The stimulation of these phagocytes, however, requires additional listerial virulence factors such as internalin (*inlA*) and haemolysin (*hylA*). Internalin (*inlA*) causes the internalisation of the bacteria; haemolysin (*hylA*) causes haemolysis of red blood cells which are both regulated by *prfA* gene. Invasiveness

associated protein (*iap*), is useful for invasion of nonprofessional phagocytic cells, cell division and intracellular uptake of *L. monocytogenes* by mammalian cells. It has also been known to produce catalase, superoxide dismutase and enables the organism to neutralize the effects of phagocytic oxidative burst. It is however not regulated by *prfA* (Todar, 2004).

A variety of food products have been involved in most outbreaks, including soft cheese (Bille *et al.*, 1990) and cooked meat products (Aguado *et al.*, 2004). These are considered of special risk due to the ability of *Listeria* to grow and survive in them (Aguado *et al.*, 2004).

1.3 Justification

L. monocytogenes is a bacterium that occurs widely in both agricultural (soil, plants and water) and food processing environments. In 1998, among populations tracked by FoodNet, listeriosis resulted in higher rates of hospitalisation than illnesses caused by any other foodborne pathogen and caused over one third of the reported deaths associated with food-borne illnesses.

The organism has been isolated in faecal samples of cattle, sheep, goats, chickens and pigs (Umeh and Okpokwasili, 2009) and water samples obtained from lakes in Nigeria (Nwachukwu *et al.*, 2010). Faeces from these animals are used as fertilizers for plants and also contaminate lakes that are consumed by these animals as food and source of drinking water, respectively.

There is paucity of information on the prevalence of this organism in fresh beef in Zaria metropolis. Listeriosis is considered a serious health problem due to the severity of symptoms and its high mortality rate. In spite of the high mortality rate and the increasing resistance of *L. monocytogenes* to antibiotics, there are limited studies on the occurrence of the organism in fresh meat in Zaria metropolis.

Due to the fact that consumption of meat has increased tremendously especially due to improved conditions of living, which reveal an upward trend (Anonymous, 2013) adequate control and inspection of meat purchased and consumed is required in a country confronted with challenges of increasing human population and shortage of animal protein.

Therefore, the purpose of this study was to determine the occurrence, distribution, antibiotic resistance and molecular detection of *L. monocytogenes* virulence genes isolated from fresh meat and offal samples in Zaria metropolis. Data on antibiograms will be useful in the empiric management of patients whereas molecular profiles will provide templates for epidemiological applications.

1.4 Research Questions

1. Is *Listeria monocytogenes* present in raw meat and offal sold for human consumption in Zaria metropolis?
2. What are the antimicrobial resistance patterns for the isolates obtained?
3. Do isolates possess virulence genes such as *hyl A*, *inl A*, *iap*?

1.5

Aim and Objectives:

1.5.1 Aim

To determine the occurrence of *L. monocytogenes* in fresh meat sold for human consumption in Zaria metropolis.

1.5.2 Objectives

Specific objectives are to:

1. determine the occurrence of *L. monocytogenes* in fresh meat and offal sold for human consumption in Zaria metropolis.
2. ascertain the antimicrobial susceptibilities of *Listeria* to commonly used antimicrobial agents.
3. detect *hylA*, *inlA*, and *iap* virulence genes in *L. monocytogenes* isolates using PCR.

CHAPTER TWO

LITERATURE REVIEW

2. 1 History of Listeriosis

When in 1924 Murray isolated Gram-positive rods from the blood of laboratory animals, i.e. rabbits, he could not assign these pathogenic microorganisms to any bacterial genus known at that time. Thus, he called this new agent *Bacterium monocytogenes* (Murray *et al.*, 1926). It can be anticipated that, even before Murray, other bacteriologists had already grown this particular bacterium without having a clear classification. In 1940 Pirie named the genus *Listeria* for catalase-positive, Gram-positive rods. This had become necessary, because by then such bacteria had been isolated in some other cases from humans (Nyfeldt, 1929), animals, as well as from food and the environment. These incidental observations did not, however, establish a broad awareness about this pathogen in the community of infectious disease specialists, microbiologists or food microbiologists.

The particular role of this type of bacteria as a pathogen was not realised until an epidemic of listeriosis in new-borns occurred in Germany in 1949. In the Institute of Pathology of the University of Halle, a peculiar entity was observed, hitherto unknown and called ‘granulomatosis infantiseptica’. A young Bacteriologist, J. Potel, was able to isolate bacteria from meconium, blood or various organs; he classified them in the genus *Corynebacterium* (Reiss *et al.*, 1951). At about the same time similar cases of

neonatal infections were observed and studied at the University of Bonn. The bacteria isolated from these lesions were examined by H.P.R. Seeliger (Seeliger, 1961), who detected the motility of these pathogens, which was not consistent with corynebacteria but rather with *Listeria*. At that time a new era of research on listeriosis started. In the following years Seeliger invested enormous efforts to inform the public about *Listeria* and listeriosis. He compiled the first overview in the book 'Listeriosis' (Seeliger, 1961), which is still the classical, nearly biblical publication.

Seeliger gathered a collection of 6000 *Listeria* isolates which he obtained from all parts of the world, the majority originating from patients or animals. A minority, however, originated from environmental sources. All these strains were similar, so that for many decades it was suggested that there is only one single species in the genus *Listeria*, namely *L. monocytogenes*.

About 90% of the isolates deriving from human patients belonged to either serovar 1 or 4. It has been observed, however, that some particular strains, mainly among members of serovars 5 and 6, were somewhat different. Rocourt *et al.* (1983) was the first to separate *L. monocytogenes* from other *Listeria* species by means of biochemical methods and later by genetic comparison. Whereas nearly all strains of *L. monocytogenes* were shown to be pathogenic, all other species including *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, and *Listeria ivanovii* were practically non-pathogenic.

Today, it is known that *L. monocytogenes* is pathogenic not only to human individuals but also to animals. *L. monocytogenes* has been cultivated from many species including

virtually all domestic, wild animals, birds and from fish. In an exemplary manner, an international group of researchers (Vazquez *et al.*, 2001; Portnoy *et al.*, 1992) characterised a gene cluster on the bacterial genome, which was responsible for the pathogenicity, a so-called pathogenicity island. The roles of several pathogenicity factors such as haemolysin (listeriolysin), phospholipases and actin activating protein were elucidated (Vazquez *et al.*, 2001).

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

Many different food items have been incriminated to transmit listeriae (Ryser *et al.*, 1999). Lactating cows can shed *L. monocytogenes* in milk as a consequence of mastitis for a rather long time. Thus, the pathogens can be present in natural foodstuff. In addition, listeriae can enter the food chain during processing. The microorganisms can be transferred by various utensils, for example the wooden trays on which food is kept. As has been documented several times, contamination may occur during the production and processing of other foods, such as salmon, in the processing plant. In consequence, the food industry has to adhere to the principles of hazard analysis and critical control

points to avoid or minimize the risk to the consumers (Ryser *et al.*, 1999). Nowadays it is well known that listeriosis is a foodborne disease.

There is scanty evidence that a mild and transient gastroenteritis may precede overt disease (Hof *et al.*, 2001). Obviously, this exposure to pathogenic listeriae is rather common, since more than 90% of adults possess immune lymphocytes. Whereas most normal, immune-competent individuals will overcome an initial attack and shedding of listeriae in faeces and is terminated after a few days, people at risk may suffer from disseminated infection. Sepsis, meningitis and/or encephalitis occur in most instances; however, many other clinical manifestations have been described. A particular disposition is imposed by pregnancy who have a 12-fold increased risk in comparison with the normal population to acquire listeriosis after consumption of contaminated food. Although the mother herself will pass through a mild, flu-like, febrile episode, the neonate usually comes down with the disease either as an early infection or develop a late post natal stage (Anonymous, 2005).

2.1.2 Microbiology of *Listeria* species

For some pathogenic bacteria, the minimum infectious dose is well established, but there is no certain figure for *L. monocytogenes* despite the considerable research undertaken since 1985 (Uyttendael *et al.*, 1997). It was estimated in the analysis of food samples implicated in outbreak to be between 3×10^1 to 1.6×10^9 CFU/gram with median of 10^5 . Although the infectious dose of listeriosis in humans is not known, host susceptibility probably influences the size of the inoculum that can cause infection.

A strong immune defence is essential to overcome a *Listeria* infection. Mackaness and his group (1969) at Saranac Lake in USA have studied the non-specific and immunologic mechanisms contributing to resistance to *L. monocytogenes*. The mouse model in particular has provided an excellent tool to study the relative role of the partners, such as macrophages and lymphocytes. T-lymphocytes are essential to enable a host to develop granulomas with a definite anti-listeric power.

In the intracellular habitat, *Listeria* can grow in the environment as well as in the extracellular space within a host. However, they belong to a group of bacteria that are able to penetrate into, survive and multiply within host cells, not only in phagocytes but also in virtually every nucleated cell of the body. By means of factors such as internalin A and/or internalin B the host cells are triggered to internalise the attached bacteria. Inside a host cell the pathogenic bacterium produces haemolysin and phospholipases and it can leave the phagocytic vacuole and enter the cytoplasm. Once in the cytosol, pathogenic strains will polymerise actin from the host cell cytoskeleton. This happens mainly at the apical tip of a bacterium so that the new actin filaments act like a driving force; the bacterium moves around in the host cell until it by chance gets under the surface of the host cell. This contact induces the host cell membrane to produce extrusions which penetrate a neighbouring host cell. The second host cell can engulf these ramifications containing the living *L. monocytogenes*. Hence, the bacterium will lyse the double cell membrane and invade the cytoplasm of the second host cell. By this mechanism of cell-to-cell spreading, the intracellular parasite avoids the host cell's defence mechanisms (Vazquez *et al.*, 2001). The intracellular habitat of *L. monocytogenes* is the consequence of an intense cross-talk between host and parasite.

Multiple virulence factors such as haemolysin (*hlyA*), phosphatidylinositol phospholipase C (*plcA*), actin polymerization protein (*actA*) and invasiveness associated protein (*iap*) and the regulatory gene *prfA* are important in the pathogenesis of *L. monocytogenes* infections. Serovars 1/2a, 1/2b, and 4b are the most commonly detected serotypes of *Listeria* spp. isolated from various types of clinical samples (Borucki *et al.*, 2003).

The hemolytic species of *Listeria* such as *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*, are associated with human pathogenicity. Both *L. ivanovii* and *L. seeligeri* are the rare causes of human infection. These two pathogens are responsible for meningitis in immune-competent adults. *L. monocytogenes* has been involved in known food-borne outbreaks of listeriosis (Tumbay *et al.*, 1988); however, there are some reports of *L. seeligeri* and *L. ivanovii* causing food-borne illness in humans (Rocourt *et al.*, 1983). *L. monocytogenes* is responsible for listeriosis which is associated with several complications including abortion, bacteraemia, sepsis, and meningoencephalitis (Vazquez *et al.*, 2001).

2.1.3 Classification of *Listeria* species

2.1.3.1 Based on different species

The recognised species of the genus are *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. marthii*, *L. rocourthiae*, *L. grayii*, *L. murrayi*, *L. fleisehmanni*, *L. coloradonensis*, *L. decoloradonensis*, *L. aquatic* sp. nov., *L. floridinensis* nov., *L. cornellensis* sp. nov., *L. gradensis* sp. nov., and *L. riparia* sp. nov.

2.1.3.2 Based on serology

Serotyping of *L. monocytogenes* has a low discriminatory power for subtype differentiation (Graves *et al.*, 2007); however, when combined with molecular methods based on DNA macrorestriction pattern analysis (Goulet *et al.*, 1995), it becomes a useful tool in epidemiological investigation. Both polyclonal anti-O antisera (O-I/II, O-V/VI, O-I, O-II, O-VI, OVII, O-VIII, O-IX) and anti-H (H-A, H-AB, H-C, H-D) were used in the determination of somatic and flagella antigen, respectively (Anonymous, 2005). The ELISA and agglutination techniques are useful means to quickly and economically serotype *L. monocytogenes* isolates and confirm the serotype uniformity of a culture before more-detailed epidemiological analyses are performed.

Other current methods used to subtype *L. monocytogenes* also include DNA fingerprinting using specific (Jersek *et al.*, 1999) or random (Mereghetti, 2002) PCR primers, ribotyping (Mereghetti, 2002; Nadon, 2001), and pulsed-field gel electrophoresis (PFGE) (Chou, 2006; Gilbreth, 2005). However, groupings based on these methods are still often compared to groupings based on serotype, using an agglutination (slide) method and subgrouping scheme developed by Seeliger and Höhne (1979).

Serotyping differentiates *L. monocytogenes* into 15 different serotypes based on the reactions of somatic (O) and flagella (H) antigens with a series of polyvalent and monovalent antisera. The serotypes are 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 5, 6a, 6b, and 7. Serotyping of *L. monocytogenes* isolates is not routinely performed outside of public health reference laboratories due to the limited availability and high cost of commercially produced antisera and inconvenience and reliability issues associated with producing one's own antisera using specific reference strains of *L.*

monocytogenes. However, because serotype designation has been correlated with virulence potential, only 3 of the 15 serotypes cause a vast majority of human listeriosis cases (Tappero *et al.*, 1995).

2.1.3.3 Based on culture and isolation methods

Laboratory Isolation and Culture method:

Isolation and characterization of *Listeria* species is done mainly by cultural, biochemical and molecular methods. A number of methods are available for the detection of *Listeria* species. However, culture is the best method, with detection power of about 10^2 CFU/ml. Samples to be examined are kept in packs on transit to the laboratory to help maintain the microbial loads as described in the compendium of methods for the microbial examination of foods (American Public Health Association, 1992). Colonies of *Listeria* species in general are small, smooth, translucent, and bluish gray when viewed in normal light, but blue green sheen is visible by oblique light. The most widely used selective media is OXA (Oxford agar) agar which was developed in 1989. Most literature recommend the use of OXA agar and either one of the following media; PALCAM (Polymixin Acriflavine, Lithium chloride, Ceftazidime Aesculin Mannitol), MOX (Modified Oxford Agar), and LPM (Lithium Chloride Phenylethanol Moxalactam medium) with aesculin and ferric iron. The principle of the media is the ability to hydrolyse aesculin by *Listeria* species. Since all *Listeria* species utilize B-D-Glucosidase, they cleave aesculin (esculinase), evidenced by blackening of the medium. In addition, different antimicrobial agents like acriflavin, nalidixic acid, cyclohexamide

are added to these media to suppress the growth of other non-*Listeria* species of bacteria and other microbial agents (Anonymous, 2005).

Various conventional methods are known for the isolation of *L. monocytogenes* and other *Listeria* spp from food and clinical samples that have gained acceptance for international regulatory purposes. All the methods differ from each other by their criteria of selecting primary, secondary and also selective agars (inhibitory compounds) in regard to the food types (Table 2.1).

Table 2.1: The types of the methods and media for isolation of *Listeria*

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

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

Reference: Manual, 2004.

2.2

Epidemiology of *Listeria* Spp.

Following its initial isolation and description in 1926, *L. monocytogenes* has been shown to be of world-wide prevalence and is associated with serious disease in a wide variety of animals, including man. The knowledge of this bacterial pathogen and the various forms of listeriosis that it causes has until recently been extremely limited, but recent advances in taxonomy, isolation methods, bacterial typing, molecular biology and cell biology have improved our knowledge.

Listeria monocytogenes, *Escherichia coli* O157:H7 and *Salmonella* spp are among the most dangerous food borne bacterial pathogens in terms of human health and disease (Olsen *et al.* 2000). Until 1960, *Listeria monocytogenes* was associated almost exclusively with infections in animals and less frequently in humans. However, in the last 30 years it has been known that it is widely distributed in nature (Vázquez-Boland *et al.*, 2001). Of the several species of *Listeria*, *L. monocytogenes* is an important cause of wide spectrum of diseases in animals and humans. Species of *Listeria* have been isolated from at least 42 wild and domestic mammals, 22 species of birds, and from others like flies, ticks, fish, crustaceans, and oysters (Todar, 2004).

L. monocytogenes is detected in different types of food and its prevalence rate differs from place to place, hygiene, food content and environmental contamination rates. It was reported by several workers and also by the European Commission to be found in raw milk, (2-12 %) (Peeler and Bunning, 1990), in pasteurised milk and milk products (2-30 %) (Buchanan *et al.*, 1993), in fresh meat (0-68%) (Mena, 2004), in minced beef (7-36%), in poultry (0-85%), in fish products (4-60%) in vegetables (Opinions 1999), salads (1-12%), and about (9%) in ready-to-eat foods (Opinions, 1999). In addition, it was also detected in other foods like RTE salads (Ieren *et al.*, 2014), cold smoked fish

(Mel *et al.*, 1994), in vacuum processed meat, (Grau and Paul, 1994) and even on the hands of food processors (Kerr *et al.*, 1993), which showed that this organism could be found everywhere and affects many animals or humans.

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

It is an exquisitely adaptable environmental bacterium capable of existing both as an animal pathogen and plant saprophyte with a powerful array of regulated virulence factors. Most cases of listeriosis arise from the ingestion of contaminated food and in the UK the disease is particularly common in ruminants fed on silage. Although a number of forms of listeriosis are easily recognised, such as encephalitis, abortion and septicaemia, the epidemiological aspects and pathogenesis of infection in ruminants remain poorly understood. The invasion of peripheral nerve cells and rapid entry into the brain is postulated as a unique characteristic of its virulence, but relevant and practical disease models are still required to investigate this phenomenon (Anonymous, 2005).

In order to elucidate some aspects of the epidemiology of listeriosis in Switzerland, 181 strains of *L. monocytogenes* isolated from humans, animals, food, and the environment

were analysed by multilocus enzyme electrophoresis at 21 enzyme loci. The clone responsible for several recent food-borne outbreaks in Switzerland and in North America (marked by electrophoretic type 1 and serovar 4b) were found frequently among strains isolated from animals. Thus, animals may represent a major source of diffusion of this clone in the environment and in food, in which it has been found only sporadically. However, two other unrelated clones (including strains belonging to serovars 1/2b and 1/2c) had often been isolated from meat but not from animals. These findings indicate that contamination of meat with *L. monocytogenes* might originate mainly from the environment in which it is processed rather than from animals themselves. This could explain the differences in the distribution of *L. monocytogenes* serovars isolated from meat and from animals, (Applied and Environmental Microbiology, 1991). An epidemic of listeriosis due to contamination of a regional soft cheese occurred in Switzerland in the years 1983 to 1987 (Bille, 1990). Using Multilocus Enzyme Electrophoresis, it was shown that despite the high genetic diversity in natural populations of *L. monocytogenes*, only two clones were responsible for this and four other outbreaks (Fleming *et al.*, 1985; Ho *et al.*, 1986; Linnan *et al.*, 1988; Schlech *et al.*, 1983). These results were confirmed and led to the hypothesis that, as with many other bacterial species, most of the clinical cases may be caused by only a few clones which either are particularly common in the environment or have an unusually high level of pathogenicity (McLauchlin, 1990).

In Africa, there are reports on prevalence of *L. monocytogenes* in different specimens. For example, in Nigeria, occurrence of *L. monocytogenes* in environmental samples and vegetables were studied and reported to be 85% from cow dung, 91.6% from soil, 73.75% from vegetables (David and Odeyemi, 2007), 3.3% from ready to eat salads (Ieren *et al.*, 2014). In Northern Nigeria, 27% mortality rate due to *L. monocytogenes*

(serovar 4) was reported (Onyemelukwe *et al.*, 1983). Outbreaks have been reported in cattle in Nigeria (Akpavie and Ikheloa., 1992) and Oni *et al* (1989) isolated *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 3a, and 4b from eight species of domestic animals including horses (68.4%), pigs (36.1%), cattle (20.8%), goats (20%), dogs (20%), free range chickens (6.8%). In Ethiopia, a study carried out in 2004, gave a prevalence rate of *Listeria* species to be 31.6% and *L. monocytogenes*, 5.1% in some foods, such as, meat, cheese, fish, pork, poultry and ice-cream (Molla *et al.*, 2004).

As a result of such environmental contaminations and poor sanitary conditions during handling of the food samples, harvesting, processing, packing, preparing or cooking times, many people could be infected and develop listeriosis. It was reported that, approximately 99% of human listeriosis appear to be food-borne. The contamination sources are animal or human faecal matters. However, animal hide is found to be the most important source of contamination than faecal matter.

After entry into the digestive tract following ingestion, the organism has a long incubation period, 1-90 days. In immuno-competent individuals, listeriosis may occur mostly after being exposed to higher doses of the organisms. However, exposure of vulnerable individuals to even low doses may end up in acquiring listeriosis (Todar, 2004). The clinical course of infection usually begins about 20 hours after the ingestion of heavily contaminated food in case of gastroenteritis, whereas the incubation period for the invasive illness is around 20 to 30 days or much longer. Similar incubation periods have been reported in animals for both gastroenteritis and invasive disease (Vazquez-Boland *et al.*, 2001)

2.2.1 *Listeria* spp in the environment

L. monocytogenes has been identified throughout the environment. The organism has been isolated from soil (Welshimer, 1960), water, and decaying vegetation (Weis, 1975; Welshimer, 1968). Weis detected *L. monocytogenes* in 21% of 779 plant and soil samples; serotypes 1/2b and 4b were most frequently found (Weis, 1975). The ability of *L. monocytogenes* to survive in the environment has been demonstrated quantitatively by Watkins and Sleath (1981), who isolated *L. monocytogenes* from samples of sewage, river water, and sewage sludge by using cold enrichment methods. They found that quantitative counts of *L. monocytogenes* from sewage sludge sprayed on agricultural land remained unchanged for at least 8 weeks. The potential implications of using faecal material as agricultural fertilizer subsequently became evident when this practice was suspected to have contributed to a large outbreak of human listeriosis in Nova Scotia (Schlech *et al.*, 1983).

2.2.2 Listeriosis in animals

Beginning with Murray and colleague's description in 1926 of disease in rabbits caused by the bacterium now known as *L. monocytogenes*, listeriosis has generally been thought of as a Veterinary disease. In mammals, *L. monocytogenes* causes abortions and "circling disease" (meningoencephalitis), and epizootics of listeriosis were observed in herds of cattle and sheep long before outbreaks of listeriosis were recognised in humans. In addition, healthy animals could be gastrointestinal carriers of *L. monocytogenes*.

L. monocytogenes has been isolated from cattle, pigs, sheep, chickens, turkeys, ducks, and a variety of other species (Seeliger, 1961). Systematic culture of stool from cows with *Listeria*-related abortions, from healthy cows in herds with listeriosis, and from

cows in unaffected herds revealed differences in the carriage of *L. monocytogenes* (Bojsen-Moller, 1972). The highest rates were found in stool cultures of animals which had aborted because of listeriosis, while healthy cows from affected and unaffected herds had lower rates of carriage.

Human disease was thought to be due to illness occurring in animals, and the animal host was considered the primary reservoir for the organism (Bojsen-Moller, 1972). Consistent with this view, conjunctivitis was reported in poultry workers who handled infected chickens (Felsenfeld, 1951), and cutaneous lesions have been reported among Veterinarians and cattle ranchers who delivered infected and aborted calves (Cain *et al.*, 1986; Owen *et al.*, 1960). In most reported cases of human illness, however, there is no history of direct contact with animals.

The idea that human listeriosis could be the result of indirect contact with infected animals focused attention on the possibility of transmission by foodborne organisms. Case reports had suggested that human listeriosis occurred after consumption of contaminated foods such as unpasteurised milk from cows known to be infected with the organism. However, *Listeria* species may be isolated frequently from the environment or from foods without a direct relation to human illness, although, initial typing systems were not sufficiently precise to suggest that an environmental strain was related to a clinical isolate. Investigation of large outbreaks of human listeriosis finally provided epidemiologic and laboratory support to confirm that listeriosis was a foodborne disease (Bille, 1990; Fleming, 1985; Linnan, 1988; Schlech, 1983).

2.2.3 Listeriosis in humans

As early as 1926, Murray suggested, that the intestinal tract might be the portal of entry for organisms causing *Listeria* infections. While most studies of nasopharyngeal cultures from healthy individuals failed to detect *Listeria* spp. several investigators have found *L. monocytogenes* in faecal specimens from healthy people (Bojsen-Moller, 1972; Gregorio *et al.*, 1975; Kampelmacher *et al.*, 1969, 1972; Lamont *et al.*, 1986), a fact consistent with the suggestion that the gastrointestinal tract is the human reservoir of the organism. Other studies from various pregnant and non-pregnant populations have identified wide-ranging estimates for stool carriage of *L. monocytogenes* among healthy adults. Kampelmacher (1969) found that 11.9% of office personnel sampled had faecal *Listeria* carriage, and slaughter house workers had a rate of 13.3%. Gregorio and Eveland (1975) found *L. monocytogenes* in 1.75% of stool samples from 400 patients hospitalised with non-*Listeria* related conditions. Differences in culture methods as well as in dietary and host factors may have accounted for differences in the prevalence of carriage. Higher rates of *Listeria* carriage have been reported when serial specimens from individual subjects were cultured, but comparing such estimates of cumulative prevalence with estimates of point prevalence is problematic.

2.2.3.1 Listeriosis of women during pregnancy

Symptoms include fever, chills, headache, backache, and discoloured urine, pharyngitis, diarrhoea, and pyelitis (inflammation of the pelvis of the kidney). These "flu-like" symptoms are the expression of *Listeria* bacteraemia. Infection of a pregnant woman leads to infection of the foetus through transplacental route or during delivery, and cause damage to the embryo resulting in abortion or stillbirth. The women may carry *L. monocytogenes* in the genital tract for some time (Marth, 1988).

2.2.3.2 *Listeriosis of the newborn*

A baby infected with *L. monocytogenes* from mother, develops variable symptoms, including respiratory distress, heart failure, cyanosis, refusal to drink, vomiting, convulsions, soft whispering, small cutaneous granulomas (Listerionomas) in the posterior pharyngeal wall, leukocytosis, development of nodules in various organs, and some may become neurodevelopmental handicaps after recovery (Marth, 1988).

2.2.3.3 *Additonal forms of listeriosis*

Meningitic and meningo-encephalitic listeriosis usually occurs in newly-born and older persons and may be fulminant with a fatality rate of about 70%. Cutaneous Listeriosis essentially produces primary skin lesions in the form of nodules, caused by direct contact with the infected animal tissues, usually affecting in farmers and Veterinarians. Septicaemic listeriosis with Pharyngitis and Mononucleosis; Symptoms include fever, severe pharyngitis, and a leukocytosis accompanied by mononucleosis. Sometimes it turns into the meningitic form of the disease. Granulomatosis Septica and Pneumonic listeriosis takes a septic course with various clinical manifestations which may develop symptoms of pneumonia, acute or sub-acute endocarditis. Focal infections result in arthritis, osteomyelitis, spinal or brain abscesses, peritonitis, and cholecystitis (Marth, 1988).

2.2.4 Patterns of disease

2.2.4.1 *Community outbreaks*

Several community outbreaks have been documented. Schlech (1983) reported a large outbreak in the Maritime Provinces of Canada which provided the first evidence for

transmission of listeriosis by foodborne organisms. The outbreak was traced to the consumption of cabbage which was fertilised with composted and raw sheep manure. In September and October 1979, in Boston due to tuna fish, chicken salad and cheese (Ho *et al.*, 1986). Fleming, (1985) reported an outbreak in Massachusetts which occurred in individuals from 30 June to 30 August 1983 with a case fatality rate of 29% due to a brand of pasteurised whole or 2% fat milk. Soft cheese was responsible for another outbreak of listeriosis, centred in Switzerland and recognised in 1987 (Bille, 1990). The outbreak was traced to a regionally produced soft smear cheese which was distributed to several countries in Europe.

An unusual outbreak of listeriosis occurred in Philadelphia, in 1987 (Schwartz *et al.*, 1989). Most (32 of 36) patients were non-pregnant adults, the majority of whom had immunosuppressive conditions or were elderly. Only 3 of the 36 patients had no immunosuppressive risk factor for listeriosis.

The interval between eating a contaminated food and onset of symptoms appears to be much longer for listeriosis than for other foodborne diseases, which typically cause symptoms from hours to a few days after exposure to contaminated food. Investigation of sporadic listeriosis is therefore quite complicated, since relevant food exposures may be those which occurred several weeks prior to the onset of disease.

Much apparently sporadic listeriosis may be the result of contaminated food sources, but temporal clustering of cases may not be recognised if the vehicle is widely distributed or if patients present to different medical facilities as is the case with raw meat.

2.2.4.2 Nosocomial outbreaks

In addition to the large community outbreaks attributed to contaminated food sources, several

Nosocomial clusters of neonatal listeriosis have been reported (Larsson, 1978). Most of the reports describe the delivery of an infant with early-onset infection followed by the diagnosis of late-onset listeriosis in one or more infants born subsequently (Simmons *et al.*, 1986). In most reports, person-to-person transmission caused by inadequate hand washing and breaks in barrier nursing technique was considered to have caused late-onset infections (Lubani *et al.*, 1987). In some reports, the affected infants had common exposure to resuscitation equipment (Simmons *et al.*, 1986), and in one hospital, inadequate cleaning of a shared rectal thermometer may have caused two cases of necrotizing enterocolitis due to *L. monocytogenes*, although transmission from person to person could not be ruled out (Larsson, 1978).

An outbreak investigation in Costa Rica suggested nosocomial clusters of listeriosis and provided the opportunity to evaluate risk factor for late onset disease (Schuchat *et al.*, 1991). This outbreak was due to contaminated mineral oil.

2.2.4.3 Sporadic listeriosis

Surveillance to determine the number of cases of disease due to *L. monocytogenes* have been difficult. Voluntary reporting to state health departments and analysis of hospital discharge data have been used to attempt to estimate the incidence of the disease (Bruce *et al.*, 1991). However, such methods have low sensitivity and are likely to underestimate the true rate of disease. To obtain a more precise estimate of the

incidence of listeriosis, the Centres for Disease Control and Prevention (CDC, 1989) performed active surveillance for listeriosis in 1986 in five states and Los Angeles County (total population, 34 million). The surveillance project detected an annual incidence of 0.7 cases per 100,000 populations. On the basis of this study, CDC estimates that 1,700 cases of listeriosis occur annually in the United States and result in 450 adult deaths and 100 foetal and postnatal deaths. The overall rate of perinatal listeriosis was 12.7 cases per 100,000 live births, but Los Angeles County had a rate of perinatal listeriosis significantly higher than that of other areas (24.3/ 100,000 births versus 7.8/100,000 births). Race-specific rates of perinatal listeriosis for both blacks and whites were higher in Los Angeles County than elsewhere. These differences could reflect true geographic differences in incidence, or they might be the result of differences in obstetric practices that could lead to enhanced diagnosis of perinatal listeriosis (Bruce *et al.*, 1991).

The incidence of listeriosis increased with age; 84% of the patients with non-perinatal listeriosis were over 50 years old, and 41% were 70 years or older (Bruce *et al.*, 1991). Persons who had eaten undercooked chicken or uncooked hot dogs were more likely to develop listeriosis. Though this epidemiologic study did not include laboratory investigation of the actual foods eaten by patients with listeriosis, the results had substantial plausibility (WHO, 1988). Inspection of the factory resulted in detection of the same strain of *L. monocytogenes* in cultures of environmental surfaces and in final container packages ready for sale (Wenger *et al.*, 1990).

Transmission by foodborne organisms may account for the largest portion of sporadic disease, but other modes of transmission may play some role in occasional cases. Although *Listeria* spp have been isolated from urethral exudates (Seeliger, 1961), there is no evidence that sexual transmission contributes to perinatal listeriosis infection. Because *L. monocytogenes* can survive refrigeration and because bacteraemia may be asymptomatic, transmission of listeriosis through blood transfusion theoretically could occur. Such transmission has been documented for *Yersinia enterocolitica* (Tipple *et al.*, 1990), but no such occurrence has been described for *L. monocytogenes*.

2.2.4.4 Invasive disease in non-pregnant adults

In 1967, Louria first described an association between listeriosis and malignant disease (Louria *et al.*, 1967), and more recent reviews suggest that most invasive listeriosis occurs in persons who are immunosuppressed or elderly (McLauchlin, 1990). Use of immunosuppressive medications for the management of malignancies and in organ transplantation has increased the immunosuppressed population and brought about increased attention to listeriosis in the medical literature (Nieman *et al.*, 1980). With the epidemic of acquired immunodeficiency syndrome (AIDS) and the resulting rapid expansion of the population at substantial risk for listeriosis, this disease may be more frequently diagnosed.

In a study of sporadic listeriosis cases which occurred in 1986 to 1987 in a geographically diverse population in the United States, 88% of non-perinatal cases occurred in persons with one or more underlying diseases (Schwartz *et al.*, 1988). Additional underlying illnesses that have been reported in association with listeriosis include sarcoidosis, chronic otitis, collagen-vascular disease, idiopathic

thrombocytopenic purpura, asthma, ulcerative colitis, and aplastic anaemia (Nieman *et al.*, 1980).

Several investigators have reported listeriosis among persons with AIDS or human immunodeficiency virus infection and have commented on the surprisingly "infrequent" occurrence of listeriosis among this population with substantial deficiency of cell-mediated immunity (Kales and Hultzman, 1990). Although it is not one of the more-common infections experienced by human immunodeficiency virus-infected patients, listeriosis does occur approximately 300 times more frequently in persons with AIDS than in the general population (Gellin *et al.*, 1989). Non-pregnant adults with listeriosis most often present with meningitis, meningoencephalitis, or sepsis. Additional syndromes include abscesses of the brain (Dee *et al.*, 1986), endocarditis (Gallagher *et al.*, 1986), endophthalmitis (Bagnarello *et al.*, 1977), osteomyelitis (Houang *et al.*, 1976), and septic arthritis (Breckenridge *et al.*, 1980).

2.2.4.5 Neonatal listeriosis

Neonatal listeriosis is divided into early and late-onset disease syndromes, similar to the pattern of the more common neonatal infections due to group B streptococci. Early-onset listeriosis, results from intrauterine infection, which can cause clinical illness in the new-born at birth or shortly thereafter. The clinical presentation of early-onset disease is most often sepsis, although granulomatosis infantisepticum, an overwhelming form of infection, occurs less frequently. This syndrome is characterised by widely disseminated granulomas in the liver and placenta as well as other organs.

In contrast to early-onset listeriosis, late-onset disease occurs several days to weeks after birth. Evidence of central nervous system infection abound (McLauchlin, 1990) and case fatality rates appear to be lower in late-onset disease than in early-onset infection. While vertical transmission from mother to foetus accounts for early-onset listeriosis, modes of transmission for late onset disease are poorly understood.

2.2.4.6 Non-invasive disease

Mild syndrome due to listeriosis presents with invasive syndromes such as meningitis and stillbirth, the association of the illness with a foodborne organism has not always been apparent. Listeriosis differs from other foodborne diseases in which non-invasive syndromes have been well characterised. One may speculate that a milder gastrointestinal illness might occur when persons without underlying immunosuppression eat food contaminated by *L. monocytogenes*. If this were the case, the numbers of cases of illness caused by *Listeria spp.* would be much greater than previously estimated.

2.3 Meat and meat products

Since 1910, the first year that statistics were compiled, Americans have been eating an average of 60 pounds of beef yearly. About 36 million cattle were inspected in 1997 alone by USDA's Food Safety and Inspection Service. This translates into 64 pounds of beef per person in 1997. Meat is generally regarded as one of the most commonly consumed and nutritious items of human diet (Fapohunda *et al.*, 1985). The domestication of cattle for food dates to about 6500 B.C. in the Middle East.

Meat is an ideal medium for the development and reproduction of micro-organisms particularly bacteria and rapid growth can be expected unless control is effected. Several micro-organisms have been incriminated in foodborne illnesses resulting from consumption of contaminated meat and meat products. These organisms include *Staphylococcus aureus*, *L. monocytogenes*, *Salmonella spp.*, *Yersinia enterocolitica*, *Bacillus cereus*, *Escherichia coli* and *Clostridium perfringens* (Saide-Alboronz *et al.*, 2005). Microbial contamination can cause spoilage of meat, reduce shelf life of meat and cause public health hazards (Rao, 1992).

The microbial contamination of carcasses occurs mainly during processing and manipulation such as skinning, evisceration, storage and distribution at slaughter houses and retail establishments (Gill, 1998; Abdalla *et al.*, 2009). Bacteria which are responsible for food borne diseases contaminate meat directly and indirectly from animal excreta at slaughter process; also they may be transferred from contact surfaces of abattoir, utensils and other equipment (Arnold International Students, 1993; Yen, 2003). The external contamination of meat constitutes a constant problem in most developing countries where they are potential sources of infection (Lawrie, 1979).

Slaughtering and the various processes involved in getting meat ready to final consumers provide opportunities for the contamination of the carcass by pathogenic bacteria (Forsythe and Hayes, 1998) so that all surfaces in contact with meat should be taken under control or kept clean to minimize the risk of bacterial contamination (Butterorth-Heineinann, 2000). Unsanitary methods spread such diseases as salmonellosis, cholera, *E. coli* food poisoning and listeriosis (Neil-Trent *et al.*, 2002).

There have been reports of *Listeria*-associated gastroenteritis following the consumption of different food types (Frye *et al.*, 2002). *L. monocytogenes* is well known for its ability to form biofilms and to establish harbourages on food-processing equipment (stainless steel, plastic and rubber surfaces), making its eradication even more difficult, which may allow the contamination of ready-to-eat food products (Carpentier and Cerf, 2011). Its presence in faeces or on the hides of food animals facilitates its entry into meat slaughter areas, onto carcasses and subsequently onto raw meat products (Peter and Rachel, 2007). *L. monocytogenes* is destroyed by cooking, but a cooked product can be re-contaminated by poor handling practices and poor sanitation.

2.4 Virulence Associated Genes

L. monocytogenes is a model organism used in studies on the pathogenesis of intracellular parasites. It is able to penetrate, multiply and propagate in various types of eukaryotic cells and is also able to overcome the three main barriers encountered in the host: the intestinal barrier, the blood-brain barrier and the placenta (Vazquez-Boland *et al.*, 2001). Once inside the host, this bacterium has the capacity to invade phagocytic and non-phagocytic cells, to replicate intracellularly, and spread directly from cell to cell, thereby escaping the humoral immune response. Each step of the infection process is dependent upon the production of virulence factors (Portnoy *et al.*, 1992): Various genes are associated with virulence in *Listeria* spp. They include:

Internalins (*inlA*, *inlB*, *prfA*,) invasion (*invA*), actin (*actA*), haemolysin (*hylA*), invasiveness associated protein (*iap*), *LmaA* etc.

2.4.1 The *iap* gene

P60 may be involved in the invasion of nonprofessional phagocytic cells and, in addition, it may have a role in cell division. Because of its possible role in invasion, the gene was named *iap* (invasion-associated protein). The *iap* gene encodes a polypeptide of 484 amino acids containing a 27-amino-acid signal sequence which yields a mature polypeptide of 47.5 kDa. The invasiveness associated protein (*iap*) gene is not regulated by regulatory gene (*prfA*) but plays a role in virulence (Kohler *et al.*, 1991).

The extracellular protein (p60, 60 kDa), is involved in intracellular uptake of *L. monocytogenes* by mammalian cells. It does not seem to be associated with the intracellular survival, It produces catalase and superoxide dismutase, which enables the organism to neutralize the effects of phagocytic oxidative burst. In addition, it produces a Zn⁺⁺ dependent protease, which is thought to act as some exotoxin and induces delayed type hypersensitivity and other cell mediated immunity responses (Todar, 2004).

Reports suggested that p60 is directly involved in binding intestinal Caco-2 cells (Park *et al.*, 2000). It was initially thought that this protein was essential for cell viability because *iap* mutations were always lethal; however, increased expression of *iap* causes cell chain shortening. The expression of p60 is controlled at the post-transcriptional level (Wuenschel *et al.*, 1993).

2.4.2 Haemolysins

Haemolysins of *L. monocytogenes* are clearly markers for virulence. However, they have a variable expression within individual pathogenic strains. Haemolytic organisms

tend to have smooth colonial morphology (Schlech, 1988). These include listeriolysins, phospholipases and haemolysin.

2.4.2.1 Hemolysins of *L. monocytogenes*

The distribution of the *L. monocytogenes* cluster is particularly noteworthy in that it is found exclusively among a subset of lineage I strains; i.e., those responsible for the majority of outbreaks of listeriosis. Expression of these genes results in the production of a haemolytic and cytotoxic factor, designated Listeriolysin S, which contributes to virulence of the pathogen as assessed by murine- and human polymorphonuclear neutrophil-based studies (Cotter *et al.*, 2008).

2.4.2.2 Production of Listeriolysin

The listeriolysin O is similar to other sulfhydryl-activated hemolysin such as streptolysin O, pneumolysin, alveolysin, perfringolysin O (Geoffroy *et al.*, 1987). Listeriolysin O is a promoting factor of bacterial growth in vivo. It is produced by virulent *L. monocytogenes* and also by *L. ivanovii* and *L. seeligeri* and these two species are also pathogenic, mostly in animals (Vazquez-Boland *et al.*, 1989). The listeriolysin O from *L. ivanovii* shows significant homology to the deduced amino acid sequence of the listeriolysin O from *L. monocytogenes*. In culture supernatants of *L. ivanovii* a sphingomyelinase and a lecithinase activity could be detected, both enzymatic activities together contributing to the pronounced hemolysis caused by *L. ivanovii* (Kreft *et al.*, 1989).

It has been postulated that the intracellular environment imposes stress conditions similar to heat shock on invading bacteria. The intracellular environment (low pH, presence of H_2O_2 and other oxidating agents) may impose a stress on the bacterial cells to which the bacteria may react by inducing stress proteins, e.g., heat shock proteins. Listeriolysin was still very efficiently synthesized in one *L. monocytogenes* strain even intracellularly and induced under heat shock conditions in another strain. Listeriolysin appears to be the only major extracellular protein synthesized under heat shock conditions; other heat shock proteins remain cell associated (Sokolovic and Goebel, 1989).

2.4.3 Internalins

There are two internalins widely known as A and B. Internalin A (85 kDa) is used to facilitate entry into epithelial cells following ingestion while internalin B (about 65 kDa) facilitates entry into a broader host cell e.g. hepatocytes. This follows ability of *Listeria* to withstand harsh acidic stomach environment which it does using stress genes such as *bsh*, *OpuCA* and *imo1421* etc.

Table 4 Major virulence proteins in *Listeria monocytogenes* (Adapted from Bhunia, 2008).

Virulence factors	Size (kDa)	Receptor	Function
Protein regulatory factor (PrfA)	27	-	Regulation of virulence protein expression.
Internalin A (InlA)	88	E-cadherin (tight junction protein)	Responsible for invasion into intestinal epithelial cells and placenta during pregnancy.
Internalin B (InlB)	65	Met (tyrosine kinase), gC1q-R/p32	Entry into hepatocytes and hepatic phase of infection.
Virulence protein (Vip)	43	Gp96 (chaperone protein)	Invasion of epithelial cells.
Listeria adhesion protein (LAP)	104	Hsp60 (chaperone protein)	Adhesion to intestinal epithelial cells.
Autolysin amidase (Aml)	102	Peptidoglycan	Adhesion to host cells.
p60 (cell wall hydrolase)	60	Peptidoglycan	Adhesion/invasion.
Listeriolysin (LLO)	58 – 60	Cholesterol	A hemolysin aids in bacterial escape from phagosome inside the cell.
Actin polymerisation protein (ActA)	90	-	Nucleation of actin tail for bacterial movement inside the cytoplasm.
Phospholipase (<i>plcA</i> – PI-PLC; <i>plcB</i> – PC-PLC)	29 – 33	-	Lyses of vacuole membrane
Metalloprotease (Mpl)	29	-	Helps synthesis of PLC.

L. monocytogenes is an invasive bacterial pathogen capable of multiplying inside many host cells, including macrophages, enterocytes and hepatocytes (Berge *et al.*, 1988). Phagocytosis also occurs in non-professional phagocytes such as enterocyte-like Caco-2 cells (human colon carcinoma cell line) (Gaillard *et al.*, 1987). This pathogen is ingested with food and penetrates through the intestine. The bacterial invasion of the host tissues during infection starts by rapid phagocytosis of the bacteria in an iron-deprived environment, which stimulates hemolysin secretion. The directed phagocytosis inhibited by cytochalasin D was only observed with the pathogenic species (*L. monocytogenes*, *L. ivanovii*), but not in other avirulent *Listeria* spp. (Gaillard *et al.*, 1987). This clearly indicates a specific interaction between invasive bacteria and enterocytes. Listeriolysin O is a promoting factor of bacterial growth *in vivo*. Electron microscopic study demonstrated that bacteria from the non-hemolytic mutant remained inside phagosomes during cellular infection, whereas hemolytic bacteria from *L. monocytogenes* were released free within the cytoplasm. This indicates that disruption of vacuole membranes by listeriolysin O-producing strains of *L. monocytogenes* might be a key mechanism allowing bacteria to escape from phagosomes and to multiply in an unrestricted fashion within cell cytoplasm (Gaillard *et al.*, 1987).

L. monocytogenes can be found frequently in the environment, and studies suggest that human exposure to *L. monocytogenes* is not uncommon, yet invasive listeriosis occurs rarely. Factors that may influence whether invasive disease will occur include the virulence of the infecting organism, the susceptibility of the host, and the size of the inoculum. Transmission of *L. monocytogenes* by food first requires penetration of the organism through the intestine (Berge *et al.*, 1988). Intracellular multiplication can

occur in various types of cells. Mackaness first showed that *L. monocytogenes* could grow inside non-immune phagocytes (Mackaness, 1962), and experimental work suggests that *Listeria* spp. can replicate in Peyer's patches (MacDonald *et al.*, 1980). Gaillard *et al.* (1986; 1987) showed in an in-vitro model using human enterocyte-like cells that *L. monocytogenes* multiplies intracellularly after internalization into vacuoles. This directed phagocytosis was observed for *L. monocytogenes* and *L. ivanovii*, the pathogenic species of *Listeria*, but not for non-pathogenic species of *Listeria* (Gaillard *et al.*, 1987).

Sword (2008) demonstrated that growth of *L. monocytogenes* in mice and host susceptibility to experimental infection were correlated with the availability of iron to the organism. In contrast, iron deprivation stimulates haemolysin secretion. Iron deprivation within the phagosome favours production of listeriolysin O, leading to disruption of intracellular membranes. Iron availability and neutral pH in the cytoplasm therefore favours bacterial replication after escape of the bacteria from the vacuole. Although listeriolysin O appears to be important in virulence, levels of haemolysin do not directly correlate with virulence in experimental infection in mice, and discrepancies between experimental results in murine and human cell lines still need to be clarified.

Host susceptibility to *L. monocytogenes* depends primarily on cell-mediated immunity, and most cases of listeriosis occur in persons with impaired cell-mediated immunity due to disease processes, medications, or pregnancy. *L. monocytogenes* was used by Mackaness in early studies that delineated the nature of cell-mediated immunity (Mackaness, 1962). Protection in mice against *L. monocytogenes* is mediated through *Listeria*-sensitized T cells, which activate macrophages. Mackaness showed that

macrophage activation depended on specific lymphocytes (Mackaness, 1969) and that anti-lymphocyte globulin suppressed immunity to *Listeria* spp. (Mackaness, 1969). The main function of *Listeria*-sensitized T cells appears to be that of attracting, focusing, and activating macrophages at infective foci (Mielke *et al.*, 1988).

In addition, the local placental suppression of cell-mediated immunity necessary to prevent maternal rejection of the placenta may contribute to susceptibility of the foetus to infection with listeriosis (Redline *et al.*, 1987). In addition to depressed cell-mediated immunity, deficiencies in immunoglobulin M and complement activity associated with the neonatal state may contribute to the propensity of infants to develop listeriosis (Bortolussi *et al.*, 1986). Local gastrointestinal factors may play an additional role in disease in adults (Ho *et al.*, 1986; Schwartz *et al.*, 1989). Outbreaks of disease caused by foodborne *Listeriae* in which the majority of non-pregnant adults were not immunodeficient (Schlech *et al.*, 1983) might conceivably involve a higher infectious dose of *Listeria* spp. than outbreaks in which illness occurred only in immunosuppressed persons (Schwartz *et al.*, 1989).

2.6 Pathophysiology

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

gastrointestinal tract is independent of Inl A and is restricted to the Peyer's patches, suggesting a predominant role for specialized phagocytic M-cells in bacterial uptake. In similar manner, in rats, intestinal translocation rates are low and independent of the Inl A, B loci, suggesting a passive process that does not involve Inl A or Inl B.

However, mice and rats are not natural hosts and furthermore, mouse and rat E-cadherin differ from human E-cadherin at an important amino acid residue that renders cells resistant to Inl A mediated invasion. In contrast to this, *L. monocytogenes* can directly invade enterocytes in guinea pigs, which are naturally susceptible to listeriosis. In transgenic mice, enterocytes expressing human E-cadherin are also susceptible to invasion by *L. monocytogenes* (Vazquez-Boland *et al.*, 2001)

L. monocytogenes is capable of multiplying extracellularly and intracellularly, within macrophage after phagocytosis or within parenchymal cells after induced phagocytosis, and then spread to hepatocytes of the liver, causing cell disruption. It is internalised by phagocytic and non-phagocytic cells and can deliver antigen to both endogenous and exogenous antigen processing and presentation pathways. *Listeria* latch onto receptors on the host cells and are then engulfed and remain as a membrane-bound vesicle, but *Listeria* soon escapes into the cytosol (Rocourt, 1983). It is also able to penetrate the endothelial layer of the placenta and thereby infect the foetus (Todar, 2004). However, this will occur only if the immune system of the individual is compromised. This is because normally the immune system eliminates the organism before it spreads by producing T-lymphocytes. Since *L. monocytogenes* multiplies intracellularly, the effective host response is cell mediated immunity, which involves both TH1 (CD4+) cells and Tc (CD8+) cells that stimulate production of TNF (Tissue Necrotizing Factor),

gamma interferon, macrophage activating factors and a cytotoxic T cell response (Todar, 2004). In general, protection against *Listeria* is mediated via lymphokine activation of T cells and macrophages and by interleukin-18 (Vazquez-Boland *et al.*, 2001).

Listeria is able to multiply at low temperature, such that they proliferate in refrigerated foods and also are actively motile by means of peritrichous flagella at room temperature (20-25⁰C), which is helpful for survival and spread in the environment. Surprisingly, they don't synthesize flagella at body temperature. Therefore in this case, the virulence is associated with the ability of the bacteria to move into, within and between host cells, by polymerization of host cells actin (gene product *act A*) at one end of the bacterium, that can propel the bacteria through cytoplasm and, helps for movement, attachment, penetration and cell to cell spread following phagocytosis and engulfment of such phagocytic and non-phagocytic cells.

Recently, evidence has been presented that Act A may also be involved in the entry of *L. monocytogenes* into eukaryotic cells, probably by recognition of an Heparan Sulfate Proteoglycan (HSPG) receptor (Vazquez-Boland *et al.*, 2001). In addition to this, they have the properties of adherence and invasion as other virulence factors.

Listeria spp. Inl A and Inl B, are proteins encoded by the *inl A* and *inl B* genes. Inl A was shown to function as an invasin, mediating bacterial internalization by these normally non-phagocytic epithelial cells (Gaillard *et al.*, 1987). The organism is thought to express D-galactose residues on its surface, so is able to attach to mannose binding receptors on the host cells. Macrophages are well known to express this receptor, such

that they simply take up the organism by induced phagocytosis. However, following this reaction the organism is able to escape from the phagosome before phagolysosome fusion occurs by releasing its toxin product called Listeriolysin (LLO), which acts as haemolysin (Geoffroy *et al.*, 1987).

Listeria spp. have other virulence factors which include those secreted as two other types of haemolysins; phosphatidyl inositol specific phospholipase C (PI-Plc) and phosphatidyl choline-specific phospholipase C (Pc-Plc), by which it can easily disrupt membrane lipids. Two, PI-Plc and Pc-Plc B are present in *L. monocytogenes* and *L. ivanovii* (Vazquez-Boland *et al.*, 2001). Phospholipase is required for efficient lysis of the secondary phagosomes formed after listerial cell-to-cell spread. In addition to these virulence factors, other listerial products have been identified that also contribute to survival within the host such as p60 (*iap*), antioxidant factors, metal ion uptake and stress response mediators (Vazquez-Boland *et al.*, 2001).

2.7

Diagnosis

Listeriosis is diagnosed through the clinical signs, culture and isolation especially from normally sterile sites such as from blood, spinal fluid and amniotic fluid. Isolation of *Listeria* spp. from non-sterile sites such as placenta and amniotic fluid in association with clinical symptoms may suggest perinatal listeriosis. Culture of *L. monocytogenes* from stool is not helpful in diagnosis, since gastrointestinal carriage of the organism may occur without clinical disease. Serologic tests do not contribute to diagnosis of listeriosis, because antibody response may not be specific (Hudak *et al.*, 1984) and because patients with culture-proven listeriosis have had undetectable levels of antibody to *L. monocytogenes* (Schlech *et al.*, 1983).

2.7.1 Molecular diagnosis

2.7.1.1 Detection by gene probes

A gene probe is a stretch of specific single-stranded nucleic acid that is enzyme- or radiolabelled and employed for the identification and detection of a complementary nucleic acid sequence in a target organism. Being a first-generation nucleic acid detection technology, the detection of *L. monocytogenes* by gene probes is precise and relatively straightforward. *Listeria* DNA is spotted onto a supporting matrix (e.g. a nitrocellulose filter or nylon membrane), hybridized with an enzyme- or radiolabelled *Listeria* species-specific gene probe (derived from 16S rRNA, heat-shock protein P60 or other protein-coding gene), and subsequently detected with an appropriate substrate (enzyme label) or by autoradiography (radiolabel) (Klinger *et al.*, 1988; Kohler *et al.*, 1991).

2.7.1.2 Detection by nucleic acid amplification

In vitro amplification of nucleic acid is a more recent addition to the genetic detection methods for pathogen identification and diagnosis. Among several elegant approaches to nucleic acid amplification, PCR was the first and remains the most widely applied technique. PCR employs two primers (usually 20–30 nucleotides long) that flank the beginning and end of a specific DNA target, a thermostable DNA polymerase that is capable of synthesizing the specific DNA, and double-stranded DNA to function as a template for DNA polymerase. The process consists of denaturation, annealing and extension of the template. The whole process is repeated 25–30 times so that a single copy of DNA template can turn into billions of copies within 3–4 h. It offers exquisite specificity, unsurpassed sensitivity, rapid turnover, and ease of automation for laboratory detection of *L. monocytogenes* from clinical specimens, in addition to its

value for identifying both cultured and non-cultivable organisms (Wang *et al.*, 1993; Manzano *et al.*, 2000; Volokhov *et al.*, 2002).

2.7.1.3 Subtyping

Similar to species-specific identification, two major subtyping approaches are in common use: phenotypic and genetic (molecular or DNA) subtyping. The phenotypic subtyping approach includes serotyping, phage typing, multilocus enzyme electrophoresis (MLEE) and esterase typing. The genetic subtyping approach encompasses pulsed-field gel electrophoresis (PFGE), ribotyping, PCR-based subtyping techniques [e.g. random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP) and repetitive element PCR (REP-PCR)] and DNA sequencing-based subtyping techniques [e.g. multilocus sequence typing (MLST)]. While the phenotypic subtyping approach occasionally suffers from low discrimination and reproducibility, the genetic subtyping approach is highly sensitive, discriminatory and reproducible (Wang *et al.*, 1993; Manzano *et al.*, 2000; Volokhov *et al.*, 2002).

2.7.2 Phenotypic subtyping methods

2.7.2.1 Serotyping

By separating *L. monocytogenes* strains into 15 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 5, 6a, 6b and 7) on the basis of serological reactions between somatic (O)/flagellar (H) antigens and their corresponding antisera (Seeliger & Höhne, 1979; Seeliger & Jones, 1986), serotyping may potentially be useful for tracking *L. monocytogenes* strains involved in disease outbreaks.

2.7.2.2 Phage typing

Bacteriophages are viruses that occur naturally in *Listeria* and other bacteria. Being host specific, bacteriophages have the capacity to lyse closely related *Listeria* bacteria independently of the bacterial species and serovar identities. Through examination of bacteriophage-induced, host-specific lysis of *Listeria* bacteria on agar plates, *Listeria* strains can be separated into distinct phage groups and phagovars, which are useful for tracking the origin and course of listeriosis outbreaks (Audurier *et al.*, 1984).

2.7.2.3 Multi-Locus Enzyme Electrophoresis

MLEE is a protein-based, iso-enzyme typing method that correlates specific protein band patterns with genotypes. For this method, soluble proteins (or bacterial lysates) from *L. monocytogenes* strains are separated by starch gel electrophoresis, polyacrylamide gel electrophoresis (PAGE), or iso-electrophoretic focusing under native conditions, followed by visualization with specific enzyme stains. Variations in the electrophoretic mobility of different enzymes (or electrophoretic types, ETs) enable differentiation of *L. monocytogenes* strains. Since multiple enzymes are present in *L. monocytogenes*, numerous ETs are often obtained (Graves *et al.*, 2007).

2.7.2.4 Esterase typing

Esterases are a class of heat-stable enzymes that hydrolyse carboxylic acid esters. Being a variant of MLEE analysis, esterase typing measures the esterase activity from the cell extracts of individual *L. monocytogenes* strains on starch gels following electrophoresis. Upon examination by esterase typing of 219 *L. monocytogenes* isolates from milk, non-dairy foods, and clinical and veterinary sources, Harvey and Gilmour (1996) detected 59 ETs.

2.7.3 Genetic subtyping methods

2.7.3.1 *Pulse Field Gel Electrophoresis*

PFGE is a highly reproducible, discriminatory and effective molecular typing method that is based on restriction fragment length polymorphisms (RFLPs) of bacterial DNA. In RFLP analysis, bacterial genomic DNA is digested with restriction enzymes to yield hundreds of fragments, which are then separated by conventional agarose gel electrophoresis to form distinctive banding patterns for individual strains, but the interpretation of RFLP results is notably tedious and technically demanding. PFGE uses selected restriction enzymes to yield between 8 and 25 large DNA bands of 40–600 kb in size, and alternating currents to cause DNA fragments to move back and forth, resulting in a higher level of fragment resolution. The plugs containing the digested DNA are transferred into an agarose gel and electrophoresed for 30–50 h with alternating currents (Brosch *et al.*, 1994, 1996). However, due to its time-consuming nature (taking 30 h or longer to perform) and its requirement for special equipment, PFGE is not widely used outside reference laboratories.

2.7.3.2 *Ribotyping*

Ribotyping is a derivative of RFLP analysis that uses a ribosomal DNA (rDNA) probe to detect the restriction fragment patterns of chromosomal DNA digested with appropriate restriction enzymes, resulting in much simpler and more consistent band patterns. Thus, only DNA fragments that contain rRNA genes are recognised (Graves *et al.*, 2007). On the whole, ribotyping is a robust, reproducible typing technique that has a similar discriminatory power to, but produces fewer bands than, PFGE.

2.7.4 Other PCR-based subtyping techniques include;

2.7.4.1 *Rapid Amplified Polymorphic DNA and Arbitrarily Primed PCR (AP-PCR)*

Both RAPD and AP-PCR use low-stringency PCR amplification with a single primer of an arbitrary sequence to generate strain-specific arrays of anonymous DNA fragments.

2.7.4.2 *Amplified Fragment Length Polymorphism*

AFLP is a modification of RFLP through the addition of adaptors to restriction enzyme-digested DNA, followed by PCR amplification and electrophoretic separation of PCR products.

2.7.4.3 *PCR-Restriction Fragment Length Polymorphism*

In contrast to AFLP, in which *L. monocytogenes* DNA is digested with restriction enzymes, linked with adaptors to facilitate PCR amplification, PCR-RFLP undertakes PCR amplification of one or more *L. monocytogenes* housekeeping or virulence-associated genes (e.g. *hly*, *actA* and *inlA*), followed by digestion with selected restriction enzymes (e.g. *HhaI*, *SacI* or *HinfI*) and separation by agarose gel electrophoresis (Wiedmann *et al.*, 1997).

2.7.4.4 *REP-PCR*

As it produces a similar level of discrimination to PFGE and ribotyping techniques, REP-PCR offers a valuable alternative for the rapid subtyping of *L. monocytogenes* strains.

2.7.4.5 DNA sequencing-based subtyping techniques

DNA sequencing of one or more selected genes is increasingly used for genetic subtyping of *L. monocytogenes*. Multi-Locus Sequence Typing focuses on multiple genes or gene fragments (e.g. housekeeping or virulence-associated genes) to determine the subtypes and genetic relatedness of *L. monocytogenes* isolates. Although, each of the subtyping procedures above represents on its own an elegant approach to the tracking of *L. monocytogenes* strains, the combined use of two or more procedures is generally more discriminatory and powerful than each applied alone.

2.7.5 Future trends of *Listeria monocytogenes* detection methods

Technology advances at a great pace and next generation assays that potentially have the capability for near real-time and on-line monitoring of multiple pathogens in foods are being developed (Jung *et al.*, 2003). The use of antibody-coated immunomagnetic beads for the capture of *Listeria* from food matrices or enrichment cultures has received considerable attention (Jung *et al.*, 2003).

DNA microarrays are composed of many discretely located probes on a solid substrate such as chemically modified glass slides. Each probe is complementary to a pathogen-specific gene sequence. PCR is used to amplify one or more genes, and subsequently the products are then hybridized to the array to identify species-specific polymorphism within one or more genes. This technology has been tested for the detection of *L. monocytogenes* in environmental samples (Call *et al.*, 2005).

Biosensors basically, are molecules of biological origin attached to a signal recognising material. When the sample comes in contact with the biosensor, the interaction will

initiate a recognition signal which is reported in an instrument. The most frequently used recognition signals include electrochemical and optical (UV, bioluminescence, fluorescence, etc.) transducers.

2.8 Biofilm formation in *Listeria monocytogenes*

A biofilm is a structured community of microbes embedded within an organic polymeric matrix that is irreversibly adhered to a surface (Davey and O'Toole, 2000). A biofilm consists of microbial cell clusters with a network of internal channels or voids in the extracellular polysaccharide and glycoprotein matrix, which allows nutrients and oxygen to be transported to the cells (Wirtanen and Salo 2005). About 85.96% of a biofilm consists of water, which means that only 2.5% of the total biofilm volume is detectable on dry surfaces. *L. monocytogenes* has been found to form biofilms on common food contact surfaces e.g. plastic, polypropylene, rubber, stainless steel and glass (Wirtanen and Salo 2005).

The ability to form biofilm is a demonstrated means of protection against environmental stresses (Davey and O'Toole, 2000; Pan *et al.*, 2006; Moretro and Langsrud, 2004). Biofilms form a protective microbial environment while permitting essential transfer processes to occur with the outside environment. Importantly, biofilm production by *L. monocytogenes* in food production and processing environments has been demonstrated and has been shown to protect *L. monocytogenes* from cleansing and sanitising agents, desiccation, starvation and other growth limiting conditions (Pan *et al.*, 2006; Moretro and Langsrud, 2004; Holah *et al.*, 2004).

Thick, complex biofilms are more resistant to stress and difficult to remove or disinfect (using sanitising agents and antimicrobials) than adhered single cells (Lappi *et al.*, 2004). It has, been demonstrated that several factors including physical and chemical properties of the substrate for attachment, characteristics of the strain, growth phase of the bacteria, growth media, temperature and the presence of other micro-organisms influence the cell attachment and biofilm formation ability of *L. monocytogenes* (Briandet *et al.*, 1999; Kalmokoff *et al.*, 2001; Norwood & Gilmour, 2001; Chavant *et al.*, 2002).

2.9

Treatment

L. monocytogenes is susceptible to a number of antibiotics in-vitro, including penicillin G, ampicillin, erythromycin, trimethoprim-sulfamethoxazole, chloramphenicol, rifampin, tetracyclines, and aminoglycosides. Although controlled clinical trials have not been performed, ampicillin or penicillin is usually recommended for invasive listeriosis. Ampicillin may be superior to penicillin (Lavetter *et al.*, 1971). Combination of an aminoglycoside to a beta-lactam antibiotic produces synergy in-vitro (Wiggins *et al.*, 1978), and this combination therapy is considered the treatment of choice. Laboratory study suggests that chloramphenicol and rifampin may each antagonize the bactericidal effect of penicillins (Winslow *et al.*, 1983). Case reports describe the effectiveness of treating patients who are allergic to penicillin with trimethoprim and sulfamethoxazole (Spitzer *et al.*, 1986); this therapy is bactericidal, and adequate drug levels are found in serum and cerebrospinal fluid (Winslow *et al.*, 1982). Although cephalosporins may be bacteriostatic against *L. monocytogenes*, treatment failures have been observed (Trautmann *et al.*, 1985), and hence treatment with cephalosporins is not recommended. Duration of therapy for listeriosis has not been standardized. Reasonable

guidelines range from 2 weeks of therapy for uncomplicated sepsis or meningitis to 4 to 6 weeks of therapy for endocarditis or non-meningitic disease in the immunocompromised host (Gellin *et al.*, 1989).

2.10 Control and Prevention

Control of *L. monocytogenes* is difficult due to its;

- (1) Widespread presence in the environment
- (2) Intrinsic physiological resistance
- (3) Ability to adapt to external stresses
- (4) Ability to grow at a wide range of temperatures
- (5) Ability to acquire antibiotic resistance
- (6) Possession of several virulence factors

Thus, it is an obligation to eliminate or minimize the presence of bacteria (Lowe *et al.*, 2001). Among the factors that affect the growth of *L. monocytogenes* are temperature, pH, atmosphere, irradiation and food additives. Kusumaningrum *et al.* (2003) reported that training and supervision to ensure proper hand washing and appropriate cleaning and sanitation procedures will reduce cross-contamination. Hazard analysis of critical control points during production, processing, sales and distribution of food, ready to eat salad and milk products should be inculcated in preventing listeriosis.

Optimum growth temperature of between 30 and 37°C is required. Temperature limits of growth are 1-45°C. Generation times in different foods ranged from 1.2 to 1.7 days at 4°C, 5.0 to 7.2 h at 13°C, and 0.65 to 0.69 h at 35°C. Heat resistance of *L. monocytogenes* in various kinds of foods has been reviewed by Mackey and Bratchell

(1989) and concluded that cooking food to an internal temperature of 70⁰C for 2 min is adequate to ensure destruction of this pathogen.

In case of sausage (made from uncooked meat), heating to an internal temperature of 62.8⁰C inactivated listeriae to undetectable levels (Glass and Doyle, 1989). But when an initial population of 5x10⁶ *L. monocytogenes* /ml was heated at 72, 82, or 92⁰C in a milk medium using test-tube method, consistent survival of a population of 10²-10³ cells/ml after 30 min was observed (Donnelly *et al.*, 1987). Heat resistance of *L. monocytogenes* did not differ significantly between pH 5.5 and 9.0 (Bradshaw *et al.*, 1985).

The induction of increased thermo-tolerance by sub-lethal heat shock (42-52⁰C) was generally not significant for *L. monocytogenes*; nevertheless, the increase was significant for *Salmonella* Typhimurium (Bunning *et al.*, 1990).

The minimum pH at which *L. monocytogenes* can grow is yet to be defined. Growth occurs between pH 6 and 9 (Seeliger and Jones, 1986). In tryptic soy broth supplemented with 0.6% yeast extract, all four strains of *L. monocytogenes* tested grew at pH 4.5. *L. monocytogenes* population declined at pH below 5 in a model broth system (Parish and Higgins, 1989). *L. monocytogenes* grew well in cabbage juice at initial pH 5.0 to 6.1, and had to be reduced to pH to 4.14 before complete inactivation occurred (Golden *et al.*, 1988). The organism is quite resistant to alkaline pH and can grow in liquid media at pH 9.6 (Doyle, 1988). Lowering of pH from 5.6 to 4.0 in clarified cabbage juice increased the rate of thermal inactivation (Beuchat *et al.*, 1986).

L. monocytogenes is quite tolerant to NaCl, being capable of growing in 10% and surviving for 1 year in 16%NaCl (Doyle, 1988). Growth of *Listeria* occurs in complex media such as nutrient broth supplemented with up to 10% NaCl and some strains have been reported to remain viable after 1 year in 16% NaCl at pH 6.0 (Seeliger and Jones, 1984). Lowering of temperature enhanced resistance to salt, the organism survived more than 100 days in 10-30% NaCl at 4⁰C. But it was affected by the constituent of the food; it could grow in cabbage juice with 2% NaCl but not in the presence of 5% NaCl. It grew in heat-sterilized unclarified cabbage juice containing less than 5% NaCl and tryptic soy broth containing less than 10% NaCl (Beuchat *et al.*, 1986).

L. monocytogenes is microaerophilic, and its growth is enhanced under decreased oxygen concentrations and with supplementation with carbon dioxide (Anonymous, 2003).

Chlorine is a common antimicrobial agent used in food industry. In one study, chlorine concentrations less than 50 ppm showed no antimicrobial effect but exposure to 50 ppm or greater free residue chlorine resulted in no viable cells (Brackett, 1987). Cells were treated in phosphate buffer containing chlorine and neutralized with 0.01 M sodium thiosulfate before counting on the TSA agar (Brackett, 1987). In another study, *L. monocytogenes* decreased rapidly in the presence of 0.5-10 ppm chlorine during the first 30 s followed by a slower decrease during the rest of the exposure time (El-Kest and Marth, 1988).

Cells were more resistant to chlorine when they were:

(A) Harvested from a 24-rather than 48-h-old culture

(B) Grown in tryptose broth rather than on a slant of tryptose agar and

(C) Washed and re-suspended using a 20mM rather than 0.312 mM phosphate buffer solution. Presence of organic matter such as peptone caused a large and rapid loss of available chlorine, but glucose or lactose had no effect. Effect of chlorine is also affected by the pH, temperature and strain (El-Kest and Marth, 1988).

Scanning electron microscopy was done to evaluate the attachment characteristics of *L. monocytogenes* on stainless steel. *L. monocytogenes* was found to produce a fibrous-like material similar in appearance to acidic polysaccharide fibrils produced by *Pseudomonas* sp., which appeared to be removed by the sanitizer solutions (chlorine or quaternary ammonium) (Mustapha and Liewen, 1989).

Deferred antagonism from diffusion assays, out of which seven among fourteen bacteriocin-producing strains were inhibitory to *L. monocytogenes*, namely *Lactobacillus* sp., *Lactococcus lactis* (2 strains, nisin), *Leuconostoc* sp., *Pediococcus acidilactici* (Pediocin PA-1), *P. pentosaceus* (2 strains, Pediocin A) (Harrison *et al.*, 1989). The bacteriocin PA-1 from *P. acidilactici* PAC1.0 was inhibitory to *L. monocytogenes* over the pH range 5.5 to 7.0 and at both 4 and 32°C. Inhibition was also demonstrated in several other food systems including dressed cottage cheese, half-and-half cream, and cheese sauce (Pucci *et al.*, 1988). Nisin (16 IU/ml) was found to have an initial inhibitory effect on growth of *L. monocytogenes* (Doyle, 1988). Bacteriocin produced by lactic acid bacteria can be used to control *L. monocytogenes* in food. When meat was inoculated with *L. monocytogenes*, the bacteriocin from *Pediococcus acidilactici* reduced the number of attached bacteria in 2 min by 0.5 to 2.2 log cycles depending upon bacteriocin concentration (Nielsen *et al.*, 1990). Presence of *P.*

acidilactici or pediocin AcH also inhibited the growth of *L. monocytogenes* in wiener exudates (Yousef *et al.*, 1991).

Some other chemical agents have been tested to inhibit *L. monocytogenes*. The action of Sorbic acid is affected by temperature and pH. At pH 5.6 and 4⁰C, the bacterium was inactivated by 0.25 or 0.3% of potassium sorbate, while at higher temperature (21 or 35⁰C) appreciable growth occurred. At 13⁰C and pH 5.0, concentrations of 0.2-0.3% potassium sorbate completely inhibited the growth (El-Shenawy and Marth, 1988). Similar effect occurred in case of sodium benzoate inactivation (El-Shenawy and Marth, 1988).

CHAPTER THREE

MATERIALS AND METHODS

3.1

Materials Used

Listeria Enrichment Broth (Oxoid, UK)

Supplements (For Broth base and Selective agar)

Listeria selective agar (Oxoid, UK)

Blood agar base (Oxoid, UK)

Mueller Hinton Agar (Oxoid, UK)

Nutrient agar (Oxoid, UK)

Tryptic Soya agar (Oxoid, UK)

Peptone water

3% H₂O₂

Oxidase

Ethidium bromide

Alcohol

Safranin

Crystal violet

Logol's Iodine

Agarose

Sugars (Mannitol, Rhamnose, Sucrose, mannose)

Incubators

UV cabinet

Autoclave

Stomacher

Antibiotic sensitivity Discs and Kirby Bauer disc dispenser

Transluminator

Refrigerator

Applied Biosystems GeneAmp 2400^R Thermocycler

Media

Reagents

Equipments

Pipettes and pipette tips
 Microgen^R 12L Identification Kit
 Filter paper and aluminium foil paper
 Test tubes
 Bijoux bottles
 Eppendorf tubes
 Conical flask (100, 250, 500 and 1000mls)
 PCR Kit, Primers and DNA Extraction Kits

Kits
 and glasswares

3.2

Study Area

This study was carried out in Zaria metropolis of Kaduna State, located within latitudes 11° 7', 11° 12' N longitude 07° 41' E. It is a medium sized city with an estimated population of 975, 228 and a growth rate 2.5% per annum. Zaria metropolis is divided into Zaria and Sabon Gari Local Government Areas (Ministry of Economic Development, 1996; Wikipedia). Fifty to seventy five percent of its working population derive their livelihood from agriculture. Agriculture in Zaria can be divided into two types: Rain- fed (from May to October) and irrigation farming in the dry season (from November to April). It is characterised by a tropical climate, a monthly mean temperature ranging from 12.8 to 36.7⁰c and annual rainfall of 1092.8mm (Agbogu *et al.*, 2006). Horticulture is the second most common agricultural activity in Zaria with vegetables being mostly produced, but in some cases fruits are sandwiched among cereals crops.

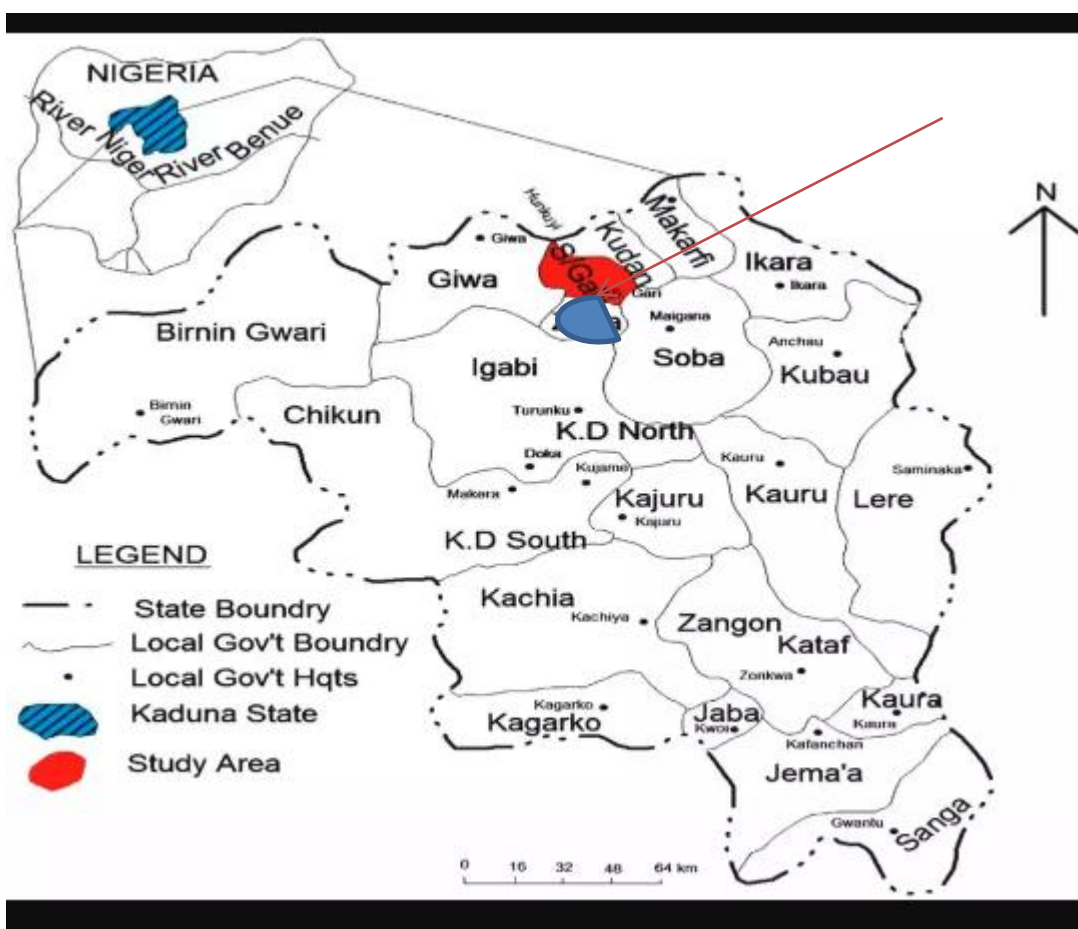


Figure 1: Map of Kaduna state highlighting the study area.

3.3 Study Design

The two Local Government Areas (Sabon Gari and Zaria) that make up the study area and the districts constituted the sampling frame. Six district areas out of 11 were selected randomly. These are: Danmagaji, Zaria city, Tudun wada, Samaru, Kwangilla and Sabon gari (Fig. 1). The target sampling locations are markets in the selected district areas.

Samples were collected using the convenience sampling technique.

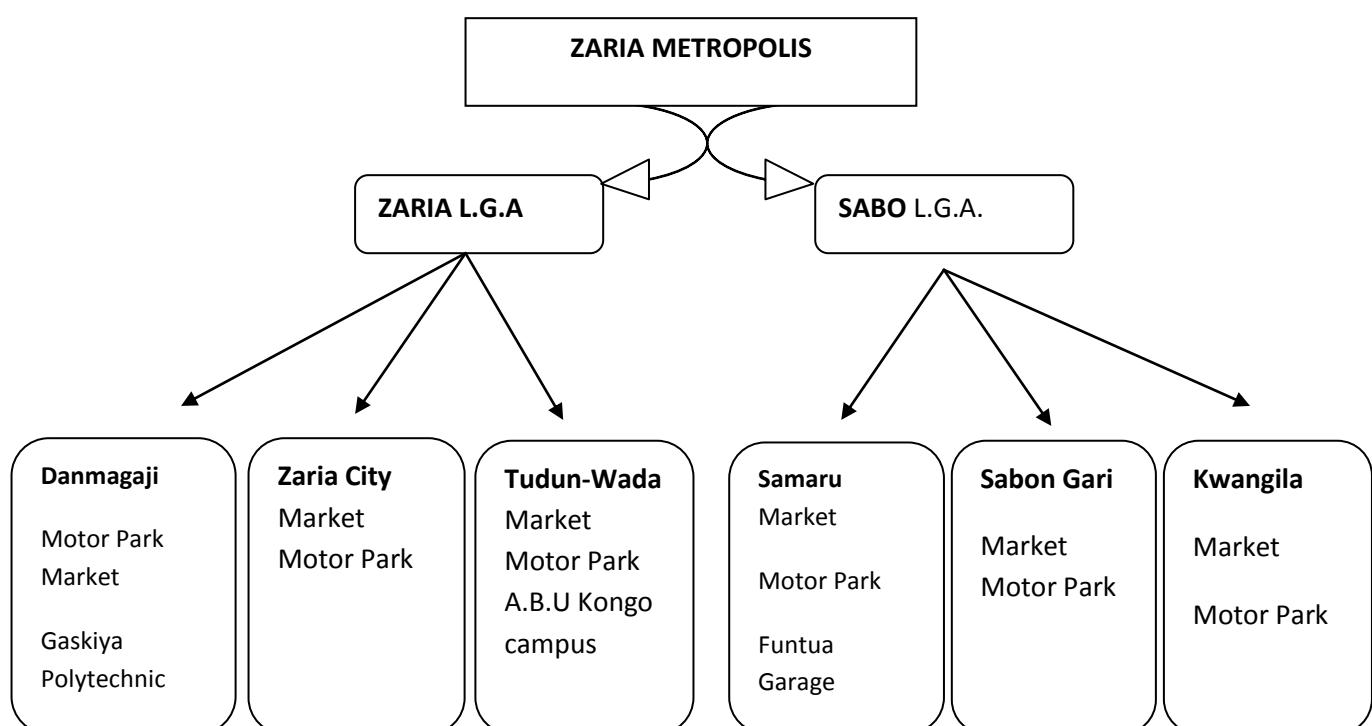


Fig. 2: Schematic representation of the sampling areas

However for this work, a total of 240 samples were taken.

3.4 Sample Collection

Two hundred and forty 240 fresh meat samples were conveniently purchased as they are normally packaged and sold. Samples were collected from all the 6 major markets in the metropolis namely Danmagaji market, Zaria city market, Tundun Wada, Samaru main market, Sabo market and Kwangila market using the convenience sampling technique. A pair of new gloves was used in handling each sample to avoid cross contamination. These samples were collected in autoclaved polythene bags and appropriately labelled after which they were placed in a flask containing ice packs for maintenance of microbial load, according to the compendium of methods for microbial examination of foods (American Public Health Association, 1992) before they were transported to the

Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria.

3.5 Sample Processing

3.5.1 Enrichment

The method of the United States Food and Drug Administration and Centre for Food Safety and Applied Nutrition (Hitchins, 2001) for the isolation of *L. monocytogenes* was used. For isolation of *L. monocytogenes*, 10g of each meat sample was weighed and aseptically enriched in 90ml of *Listeria* enrichment broth (Oxoid, UK) containing selective *Listeria* enrichment supplement (SR0141E, Oxoid, UK). The mixture was homogenised in a stomacher (Coldworth Stomacher, Seward, London) for 2 minutes and incubated for 24hrs at 37°C in a conical flask. *Listeria* species were isolated on *Listeria* selective agar (Oxoid) with *Listeria* enrichment supplements (SR0141E, Oxoid, UK) and identified using the following conventional biochemical tests: Gram staining, catalase, aesculin hydrolysis, oxidase, sugar fermentation tests (maltose, sucrose, mannitol and rhamnose) and haemolysis on 5% sheep blood agar.

3.5.2 Identification of *Listeria* species

A loop full of enriched sample was streaked on *Listeria* agar containing *Listeria* selective supplement (SR0141E, Oxoid, UK) and incubated at 37°C for 24-48hrs and observed for growth of colonies. Thereafter, plates were examined for brownish-black colonies with a raised halo typical for *Listeria* specie. Repeated sub-culturing of isolates was done to get pure colonies which were stored on nutrient agar. Thereafter, selected representative colonies were further identified to species level based on haemolytic patterns and biochemical analysis.

3.5.3 Biochemical tests

Biochemical tests carried out included; catalase, oxidase, motility; fermentation of rhamnose, mannitol, maltose and sucrose. Typical colonies of different isolates were transferred to Nutrient agar plates and incubated for 24hours. Colonies from the TSA plates were preserved on TSA agar slants and used for further tests.

3.5.4 Identification of *Listeria* by the Microgen^R 12L system

Commercially available biochemical test strips (Microgen^R 12L) (Oxoid) for the identification of animal, human and environmental strains of importance were used according to the manufacturer's instructions as a confirmatory test for *L. monocytogenes*. All isolates were sub-cultured on TSA at 37°C for 24 hours. A single colony was picked using a sterile inoculating loop and thoroughly dissolved into the broth provided in manufacturer's kit.

Wells were appropriately labelled with codes corresponding with each isolate. The covers were removed and 100µl of suspension containing the organism were inoculated into impregnated wells according to manufacturer's instructions using disposable sterile pipette tips for each sample after which they were then covered again and incubated for 24hours at 37°C. A drop of haemolysin reagent was added to well 12 of each sample immediately after inoculation. Colour changes were observed and compared with colour chart provided by the manufacturer. The colour changes were documented as numbers and the summation of which was inputted into the Microgen^R 12L identification software on a computer system which gave the interpretation and the various percentage probabilities of the suspected organisms. The contents of the wells are, Esculin,

Mannitol, Xylose, Arabitol, Ribose, Rhamnose, Trehalose, Tagatose, Glucose-1-phosphate, M-D-Gluc, M-D-Man and Haemolysin.

3.6 Antibiotic Susceptibility Testing

Listeria monocytogenes (eight isolates) were inoculated into Tryptic soya broth and incubated at 37°C aerobically using the disc diffusion method based on the protocol of Bauer *et al* (1966). The turbidity of the actively growing broth culture was adjusted with normal saline to obtain turbidity optically comparable to 0.5 MacFarland standards. A sterile swab was then used to evenly spread suspension on the surface of Mueller – Hinton agar. Antibiotic discs, namely; Chloramphenicol C (30µg), Erythromycin E (15µg), Nitrofurantoin F (300µg), Ciprofloxacin Cip (5µg), Tetracycline TE (30µg), Ampicillin AMP (10µg), Cefexime CEF (5µg), Gentamicin CN (10µg) and Sulphamethaxazole/trimethoprim SXT (25µg), Kanamycin K (30µg) and Amoxillin/Clavulanic acid AMC (30µg) were placed onto the medium with a disk dispenser (Oxoid) and incubated for 18-24 hours. The zones of inhibition that were observed in disk diffusion were measured using a transparent ruler and the antimicrobial susceptibility testing standards according to the Clinical and Laboratory Standards Institute (CLSI) guidelines was used to derive a correlation between the zones of inhibition diameters measured to the nearest millimetre and interpreted as resistant, intermediate or sensitive according to the CLSI (2011).

3.7 DNA Extraction

Two colonies of each isolate were picked, inoculated into 5ml of Luria Bertani (LB) (prepared) and incubated at 37⁰C for 24 hr. About 1500µl of the broth culture of each

of the eight isolates tested was dispensed into sterile Eppendorf tubes and centrifuged at 14000rpm for 2mins to obtain bacterial cells, the supernatant was discarded, 400µl of lysis buffer was added to the tube and the contents was thoroughly mixed and incubated at 70⁰C for 20mins. The tube and its contents were centrifuged for 2 minutes at 14000rpm. The supernatant was then transferred to a spin column and centrifuged after which 300 µl of wash buffer I (Qiagen) was added and the tube was centrifuged at 14000rpm for another 2mins. Subsequently, 300 µl of wash buffer II was added and the tube was centrifuged at 14000rpm for 2mins. The flow through from the collection tube was discarded and this step was repeated. The spin column was centrifuged empty to remove excess buffer. The spin column was then transferred to clean Eppendorf tubes, 50 µl of T.E elution buffer was then added to the column matrix, and this was allowed to stand for a few minutes before it was centrifuged at 14000rpm for another 2 minutes. This step was repeated with the addition of 30 µl of the elution buffer and the pure DNA was collected in the Eppendorf tubes. The extract was then run on agarose gel to confirm for the presence of DNA.

3.8 PCR Amplification

Amplification of the targeted fragments of the virulence specific genes (*hlyA*, *inlA* and *iap*) was done by PCR using reverse and forward primers (D1-Forward; 5' ACAAGCTGCACCTGTTGCAG 3'; D1-Reverse; 5' TGACAGCGTTGTTAGTAGCA 3' for *iap*, D2- Forward 5' CCTAAGACGCCAATCGAA 3' D2- Reverse 5' AAGCGCTTGCAAGTCCTC 3' for *hlyA* and D3- Forward 5' AGATCTAGACCAAGTTACAACGCTTCAG 3' D3-Reverse 5' TAATATCATTTGCTGTTTTATCTGTC 3' for *inlA*) with expected amplicons of 131bp, 702bp and 232bp respectively. The mix for the reaction contains: Taq DNA

polymerase, Reaction Buffer, dNTPs (dATPs, dGTPs, dTTPs, dCTPs), MgCl₂. Amplification was carried out using Applied Biosystems GeneAmp 2400 Thermocycler. Each reaction consisted of 50pmol of each primer and 5 µl of DNA template with 0.5 units of Taq polymerase. PCR cycling conditions were as follows; initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55-68°C for 30sec and extension at 72°C for 1 minute with an increment of one sec per cycle for the denaturation and extension segment after which the product was cooled and held at 4°C.

3.9 Electrophoresis

The product size was resolved using electrophoresis through 2% agarose gels containing ethidium bromide and visualized on a UV trans-illuminator. During this experiment laboratory control strain of *L. monocytogenes* ATCC 19117 was used as a positive control and Dnase free water as negative control.

3.10 Data Analysis

The data obtained were presented in the forms of tables and charts. Chi-square was used to test association between variables and values where $P \leq 0.05$ were considered significant. The analyses were done using Statistical Package for Social Sciences (SPSS version 20).

CHAPTER FOUR

RESULTS

4.1 Occurrence of *Listeria* species

From the total of 240 samples examined, 8 isolates of *L. monocytogenes* were obtained. Other species isolated were 6 *Listeria ivanovii*, 13 *Listeria innocua* and 39 *Listeria grayi* giving a total of 66 *Listeria* isolates based on conventional biochemical tests, giving an overall prevalence rate of 27.5% (Table 4.1) for *Listeria* species. When the 8 *L. monocytogenes* were subjected to identification using the Microgen^R 12L commercial test kit system, only one was identified as *L. monocytogenes* with percentage probability of 99%.

The raw meat from Tudun-wada market had the highest isolation rate of 6.7%, followed by Zaria city, 5.8%, Kwangila market, 5.4%, Sabo market 4.6%, Dan-magaji 2.9%, while Samaru market yielded the least with 2.1%. Out of the 8 *Listeria monocytogenes* isolates, 5 were recovered from Tudun wada, one each from Samaru, Sabon gari, and Zaria city; but none from Dan magaji and Kwangila. There was no statistically significant ($P>0.05$) association between *Listeria* species and the location from which they were obtained. Based on the various meat parts sampled, the female reproductive genitalia had 36%, followed by beef, 28.2%, liver, 23.1% and intestines, 21.2% (Table 4.2).

4.2 Susceptibility of *Listeria monocytogenes* Isolates to Commonly Used Antimicrobial Agents

All the 8 *L. monocytogenes* isolates were found to be resistant to one or more of the 11 antimicrobial agents tested, with high frequency of resistance to beta-lactam antimicrobial agents. Resistance were also observed with ciprofloxacin, gentamicin, chloramphenicol and Nitrofurantoin (Table 4.3).

Table 4.1: Occurrence of *Listeria* species in raw meat in Zaria metropolis (n=240)

<i>Listeria</i> species.	No. of isolates	Prevalence
(%)		
<i>L. monocytogenes</i>	8	3.3
<i>L. ivanovii</i>	6	2.5
<i>L. innocua</i>	13	5.4
<i>L. grayi</i>	39	16.3
Total	66	27.5

Table 4.2: Occurrence of *Listeria* species in different types of raw meat in Zaria

Sample Type	No Tested	No +ve for isolates
1. Beef	156	44
2. Reproductive Tract (Female)	25	9
3. Liver	26	6
4. GIT	33	7
Total	240	66

Table 4.3: Susceptibility of 8 *L. monocytogenes* from fresh meat to 11

antimicrobials

S/Number susceptible	Antimicrobial agent	Number (%) of
1.	Gentamicin	7 (87.5)
2.	Ciprofloxacin	5(62.5)
3.	Kanamycin	5 (62.5)
4.	Chloramphenicol	4 (50)
5.	Nitrofurantoin	4 (50)
6.	Sulphamethaxazole / Trimethoprim	4 (50)
7.	Erythromycin	3 (37.5)
8.	Amoxycillin/Clavulanic acid	2 (25)
9.	Tetracycline	1 (12.5)
10.	Cefexime	1 (12.5)
11.	Ampicillin	0 (0)

There were 6 antimicrobial resistance patterns with C,AMP,AMC,TE,F,SXT,CFM,E and AMP,AMC,CFM having a frequency of 2 each, and the other four i.e. CN,AMP,K,AMC,SXT,CFM,E; AMP,TE,F,CFM,E AMP,K,TE,CFM; AMP,AMC,CFM occurring only once.

4.3 Multiple Antibiotic Resistance (MAR) Index Profiles of *L.*

***monocytogenes* Isolates from Raw Meat in Zaria Metropolis**

All the *Listeria monocytogenes* were resistant to at least 3 out of the 11 antimicrobial agents tested. However, all the isolates had MAR index>0.2 but majority of the isolates were susceptible to aminoglycosides and ciprofloxacin (Table 4.4).

4.4 Amplification of Virulence Genes from the Isolates

The *iap* gene was detected in five isolates, one isolate harboured both *iap* and *inl A* gene, and the *hyl A* was not found in any of the isolates (Fig. 3).

Table 4.4: Multiple Antibiotic Resistance (MAR) Index for *L. monocytogenes* from raw meat in Zaria metropolis.

S/no (a/b)	Isolate	Number of Antibiotics to which isolate was resistant to (a)	MAR index
1	Sm1	5	0.46
2	Sb77	8	0.73
3	Zc156	8	0.73
4	Td5	8	0.73
5	Tdn6	3	0.27
6	Tdn7	3	0.27
7	Tdn9	7	0.64
8	Tdn40	4	0.36

b = Total no. of antibiotics used; 11

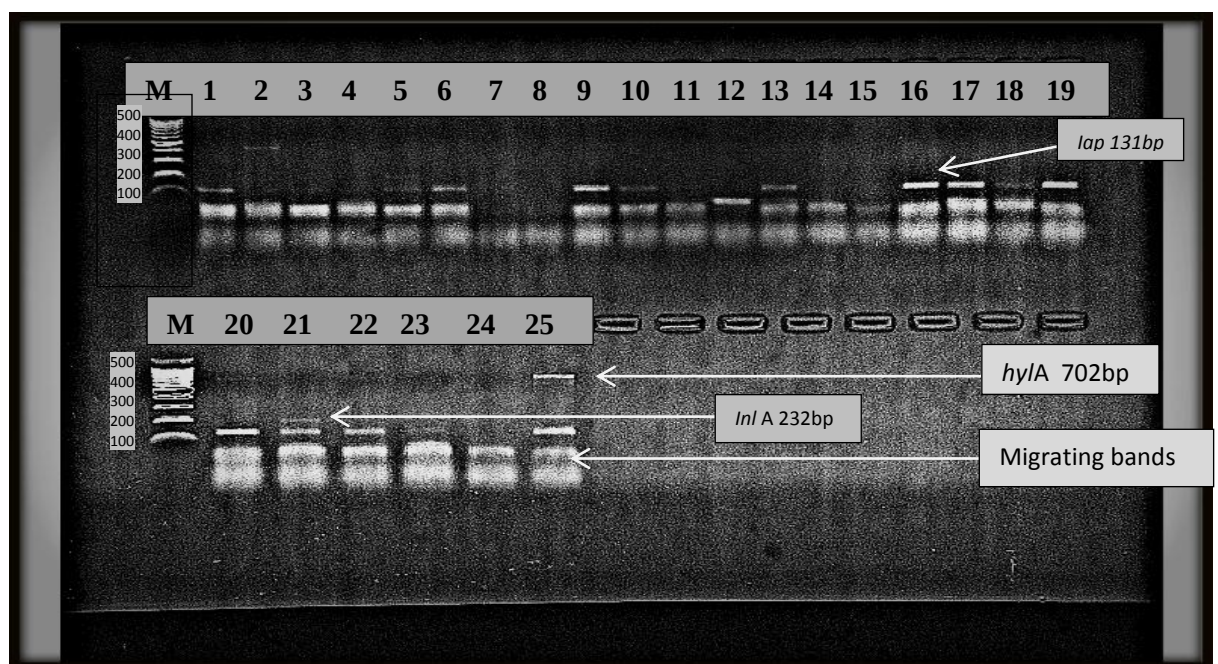


Figure 3: Multiplex PCR showing detection/amplification of the *iap* and *inl A* genes.

L. monocytogenes showing virulent genes. Lanes 9, 16, 17, 19 and 22 were isolates that amplified *iap* gene while only lane 21 detected *iap* and internalin *inlA* gene. Molecular markers are 100-bp apart. Lane M is the molecular weight ladder, 24 is negative control and 25 is the positive control.

CHAPTER FIVE

5.1

Discussion

Out of the 240 raw meat samples tested, 66 were contaminated with *Listeria* species of which 8(3.3%) were *L. monocytogenes*; other species detected were *L. ivanovii* (2.5%); *L. innocua* (5.4%) and *L. grayi* (16.3%). In this study, raw meat was found to be contaminated with *Listeria* organisms, including *L. monocytogenes*. An overall prevalence of 27.5% was obtained for all *Listeria* species. This work is similar to the work of Chukwu *et al.*, (2006) who obtained a total prevalence of 21.5% from dry smoked fish, Eruteya (2014) who obtained 33.75% from raw cow and goat meat, Firehiworth (2007) who obtained 27.4% from food samples. Although Mena (2004) had reported a prevalence of between 0-68%, other works carried out revealed an upward trend. This was observed when Ikeh (2010) obtained a prevalence of 80% while David and Odeyemi (2007) obtained 78.1% from environmental samples. These differences may be due to the differences in isolation methods, sample differences, sample sizes and environmental factors.

The specific occurrence rate obtained for *Listeria monocytogenes* was 3.3%. This is similar to the work done by Ndahi (2014) who obtained a prevalence of 3.9% in raw meat, Yucel (2005) who got an occurrence of 5.2% in meat and Ehizibolo (2007) who reported 9.2% *Listeria monocytogenes* from smoked fish in Jos, Plateau state. Other works done, Rocourt (1997) and Firehiworth (2007), revealed a higher occurrence of the organism with prevalence rates of 50% and 68.3% in fresh meat respectively. This might be attributed to the differences in location, different methods of processing, handling, culture and isolation methods. The higher rate of isolation of *Listeria* species from raw meat may be due to the fact that it has not been subjected to heat treatment and no condiments added that could either kill or suppress the growth of these organisms (Ndahi *et al.*, 2014). This study shows that meat samples may have been exposed to poor sanitary conditions, during slaughtering, processing or at point of sale.

Microgen^R 12L identification kit did not totally agree with the findings of this study based on biochemical identification as only one was confirmed as *L. monocytogenes* out of 8 isolates obtained from biochemical tests. This could mean that commercially available kits for microbial testing and confirmation require validation in our environment.

Based on the sampling locations, the highest percentage of *L. monocytogenes* isolates was obtained from Tudun Wada (6.7%). However, there was no statistically significant ($P>0.05$) association between the sampling location and the isolation of *L. monocytogenes*. This may be due to biofilm formation on equipments used in processing and sales of meat, hygienic practice by vendors and source of water.

The antimicrobial susceptibility testing of 8 *L. monocytogenes* isolates to 11 antimicrobial agents showed that 7 out of all the 8 isolates (87.5%) were susceptible to gentamicin, which is similar to the findings of Adetunji and Tajudeen (2011), Ennaji *et al.* (2008), Ndahi *et al.* (2014) and Aurelli *et al.* (2003) who all reported good activity to aminoglycosides. However, all the isolates were resistant to a minimum of 3 and up to 8 antimicrobial agents. The study of Ennaji *et al.* (2008), Aurelli *et al.* (2003), Morobe, (2009) and Chukwu *et al.* (2006) reported that ampicillin showed good activity against *L. monocytogenes*, which was entirely different from what was observed in this study, in which all the isolates were resistant to this beta-lactam antimicrobial agent, but agrees with the work of Ndahi *et al.* (2014). David and Odeyemi *et al.*, (2007), reported good activity with erythromycin which was not the case with this study as it shows low activity against the organisms. Adetunji and Tajudeen (2011), also reported resistance to tetracycline which was also observed in this study. The resistance to multiple antibiotics may be due to indiscriminate use of antimicrobial agents in the study area. The high rate and wide range of resistance to antimicrobial agents revealed abuse of antibiotic usage in cattle. The public health significance of these findings is that the resistant strains from meat tables may find their way into human population through food chain and occupational exposure (Adetunji and Tajudeen, 2011).

There were six antimicrobial resistance patterns observed for *L. monocytogenes*. All the isolates showed multiple resistance patterns with an increasing frequency from ampicillin, cefixime, tetracycline, amoxicillin and clavulanic acid, erythromycin, sulphamethoxazole/trimethoprim, nitrofurantoin, chloramphenicol, kanamycin, ciprofloxacin and gentamicin. There have been reports of acquired multiple resistance in

L. monocytogenes mediated by self-transferable plasmids (Charpentier and Courvalin, 1999).

Since all the *L. monocytogenes* isolates showed resistance to multiple antimicrobial agents and all had MAR index greater than 0.2, the implication could be that there is abuse of antibiotics in the environment. The cost of treatment would increase when humans are infected with these zoonotic pathogens, as reported by Lungu *et al.* (2011). It may also be possible that the organisms are acquiring self -transferrable plasmids for antimicrobial resistance from organisms of either environmental or clinical origin.

Four of the isolates had exactly the same resistance patterns to the antimicrobial panels of drugs tested. This could be an indication of genetic relatedness and possibly sharing of genetic materials like plasmids which makes antibiotic resistance well-coordinated and effective (Brichta-Harhay *et al.* 2011).

The detection of *iap* and *inl A* virulence genes in multidrug- resistant zoonotic bacteria should be of great concern to clinicians in treatment of patients infected by such an organism. The detection of such genes implies that the organisms are virulent. The fact that such an organism was isolated from raw meat product raises the possibility of their occurrence in other food products of animal origin such as milk or milk products.

Raw meat and meat products in Zaria, Nigeria, were found to be contaminated with *L. monocytogenes*, which displayed resistance to several antimicrobial agents. The antimicrobial resistance patterns of the organisms may be suggestive of the extent of misuse of antibiotics in Medical and Veterinary practices. Similarly, the rate of

contamination of raw meat with *Listeria* species may be an indication of human and environmental contamination and should be regarded as being potentially hazardous to consumers.

Finally, this study has been able to establish the occurrence of *Listeria monocytogenes* in fresh meat samples and that these isolates possess the *inlA* and the *iap* virulence associated genes.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1

Conclusion

The objectives of this study were to determine the occurrence of *Listeria monocytogenes* and to evaluate the susceptibility of the organisms isolated to commonly used antimicrobial agents. The findings of this study showed that *Listeria monocytogenes* was present in fresh meat and offal samples in Zaria metropolis. The isolates had multiple antimicrobial resistance and possessed virulence genes *inlA*, and *iap*. This is of public health significance.

Transmission by foodborne route is now recognised as causing both epidemic and sporadic listeriosis. Continued study of dietary risk factors associated with listeriosis is needed in order to develop dietary recommendations for the expanding population at increased risk of disease. Current research application of new molecular methods to the study of *L. monocytogenes* may improve the ability to diagnose pregnancy-associated

disease and permit the rapid detection and control of *L. monocytogenes* in the food supply.

In conclusion, raw meat and meat products in Zaria, Nigeria, were found to be contaminated with *L. monocytogenes*, which displayed resistance to several antimicrobial agents. The antimicrobial resistance patterns of the organisms may be suggestive of the extent of misuse of antibiotics in Medical and Veterinary practices. Similarly, the frequency of contamination of raw meat with *L. monocytogenes* species may be an indication of human and environmental contamination and should be regarded as being potentially hazardous to consumers.

6.2

Recommendations

Based on the results of this study, the following recommendations are hereby made:

- (i) There is a need for a well-organized continuous surveillance system in Zaria, Nigeria to determine the occurrence of *Listeria* in various foods of animal origin and their public health significance.
- (ii) There is need also to develop policies and locally viable practices for safer handling and processing of meat and meat products so as to maintain its benefits for food supply and livelihoods while reducing the health risks associated with its consumption.
- (iii) There is need to create awareness on molecular methods in rapid detection of *Listeria monocytogenes* that are specific at the species level which will have important applications in the food industry.

- (iv) There is need for the collaborative efforts of food producers, consumers, handlers and the government to be aware of and to disseminate useful information as to the occurrence, prevention and control for this disease.
- (v) Strict laws concerning antibiotic use and availability should be made and adhered to by relevant authorities.

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Appendix

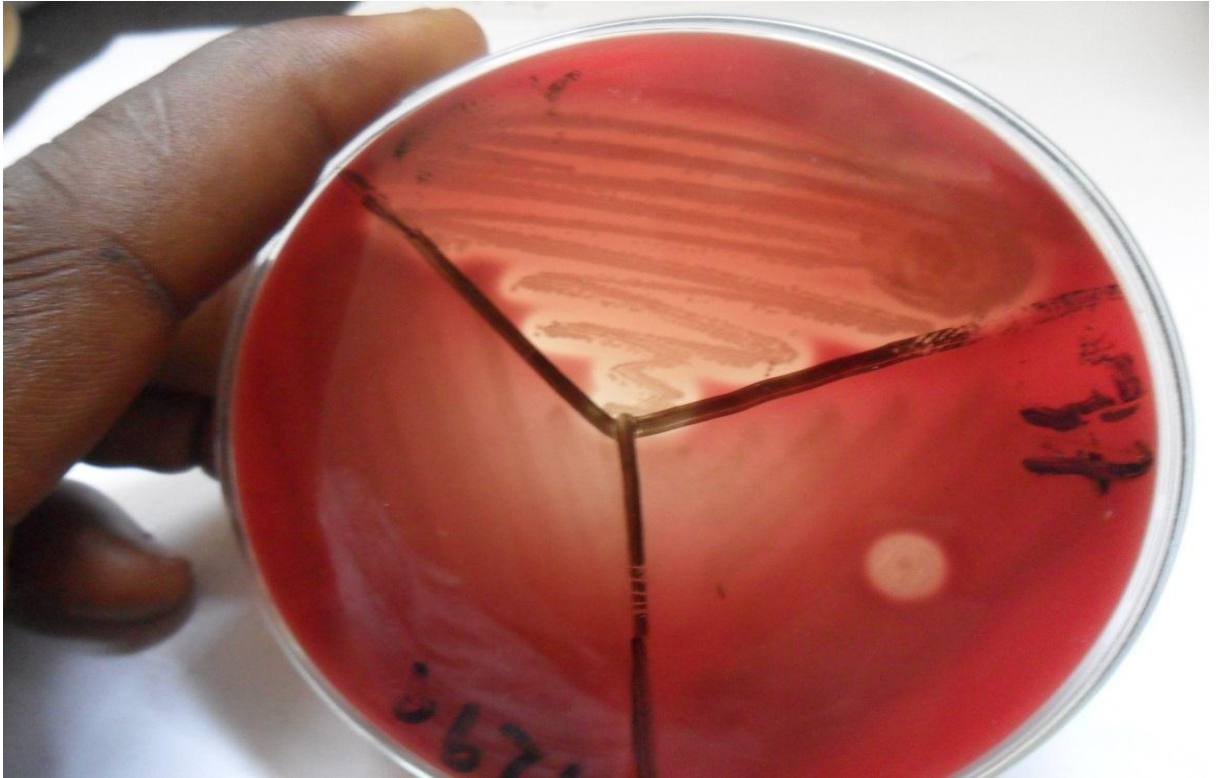


Plate I: *L. Monocytogenes* showing B-haemolysis

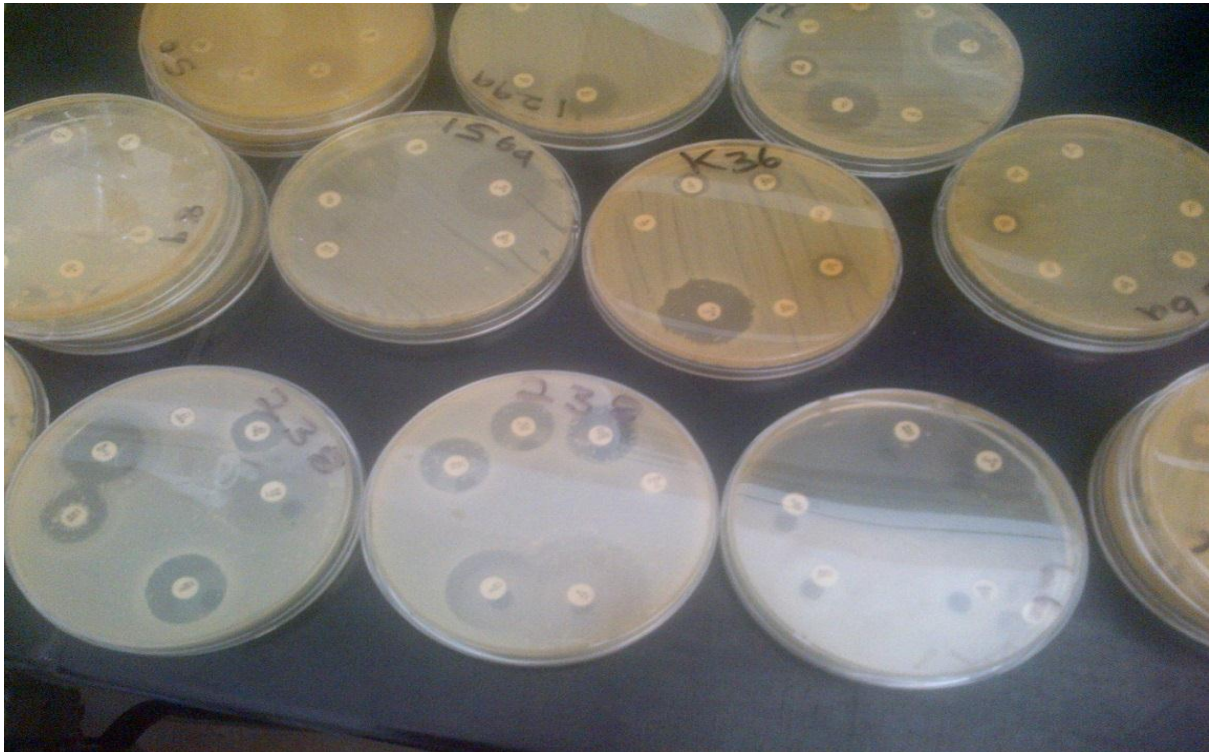


Plate II: Antimicrobial susceptibility pattern of *L. Monocytogenes* showing zones of inhibition



Plate III: Listeria Selective Enrichment Selective Supplement

