PREVALENCE, MOLECULAR CHARACTERIZATION AND ANTIBIOTIC SENSITIVITY OF *LISTERIA MONOCYTOGENES* ISOLATED FROM FOODS OF ANIMAL ORIGIN IN NAIROBI AND ITS ENVIRONS, KENYA

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DECLARATION

This thesis is my original work and has not been presented for a degree or any other award in any other University.

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DEDICATION

I dedicate this thesis to my loving wife Adele and my sons Jayden and Nathan for their endless support through the entirety of my study. May the Almighty God bless you abundantly.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immune Deficiency Syndrome
BAM	Bacteriological Analytical Manual
CAMP test	Christie Atkins Munch Petersen test
CDC	Centers for Disease Control and Prevention
CFSPH	Centre for Food Security and Public Health
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
ISO	International Organization for Standardization
KNBS	Kenya National Bureau of Statistics
MVM	Merck Veterinary Manual
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
rRNA	Ribosomal ribonucleic acid
USDA	United States Department of Agriculture
WHO	World Health Organization
LLO	Listeriolysin O
PI-PLC	Phosphatidylinositol specific phospholipase C
PC-PLC	Phosphatidylcholine specific phospholipase C
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

REP- PCR	Repetitive Extragenic Palindrome-Polymerase Chain Reaction
PBP3	Penicillin Binding Protein 3
PALCAM	Polymyxin Acriflavine LiCl Ceftazidime Aesculin Mannitol Agar
MOX	Modified Oxford Agar
LPM	Lithium Chloride Phenyl ethanol Moxalactam Agar
ORF	Open Reading Frame
° C	Degrees Centigrade

ABSTRACT

Food borne infections are an important public health concern worldwide with most being caused by pathogens that are zoonotic in nature. Among the most common food borne infection is listeriosis, caused by *Listeria monocytogenes*, a bacterium that is widely distributed in nature and which has been isolated in a wide array of foods. It mainly affects immunocompromised individuals including pregnant women, neonates and the elderly. Currently, in Kenya, there is no published data on the presence of this organism in ready to eat foods. The aim of this study was therefore to determine the prevalence, serotypes, virulence factors, genetic relationship and antibiotic susceptibility of *L. monocytogenes* from ready to eat meat products and milk products in Nairobi and its environs. A total of 570 samples; 350 milk products and 220 meat products were collected from selected retail markets in the study area. Isolation and identification was done as per the Bacteriological Analytical Manual protocol, and out of the 570 samples, 49 (8.59%) tested positive for *Listeria spp* after amplification of a 370 bp region of the *prs* gene. Twenty-one (42.8%) of these isolates were from milk products namely; milk powder (4.76%), short life pasteurized milk (4.76%), long life pasteurized milk (14.29%) and pasteurized milk from dispensing machines (76.19%). The rest, 28/49 (57.2%) were isolated from meat products namely; ham (7.14%), brawn (46.43%), polony (28.57%), salami (3.57%) and ready to eat meat bites (14.29%). Speciation of the *Listeria* isolates was done through multiplex PCR and of the 49 isolates, 22 were confirmed as *L. monocytogenes* through the amplification of a 509 bp region of the *Lmo1030* gene. Of these *L. monocytogenes* isolates, 77.27% were from milk products while 22.72% were from meat products. The highest prevalence, 68.18 % was from dispensed milk while the lowest, 4.54% was from short life milk, long life milk and ham. Of the other 27 *Listeria spp* isolates, two were identified as *L. welshimeri* by amplification of a 281 bp region of the *scrA* gene while three were identified as *L. innocua* by amplification of a 749 bp region of the *Lin0464* gene. The rest of the isolates, (22/27) were unidentified *Listeria spp*. The overall prevalence of *L. monocytogenes* was 3.86% (22/570). Molecular serotyping of the 22 *L. monocytogenes* isolates showed that 95.45% of the isolates carried both the *ORF 2110* and the *ORF 2819* genes characteristic of serotypes 4b,4d and 4e. A majority of these isolates (68.18%) were from milk collected from dispensing machines, two isolates from polony and an isolate each (4.55%) from long life milk, short life milk, brawn and ham. The remaining isolate (4.55%) carried the *ORF2819* gene only, characteristic of serotype 1/2b, 3b, 4b, 4d, 4e and 7 and was isolated from brawn. All the isolates possessed *hlyA, inlA, inlC, iap* and *actA* virulence genes. Almost all the isolates (21/22) had the *inlJ* gene while *inlB* was only detected in 10/22 (45.45%) of the isolates. All *L. monocytogenes* were resistant to penicillin, 9.09% resistant to erythromycin and 4.54% resistant to sulfamethoxazole trimethoprim. All isolates were susceptible to gentamycin, chloramphenicol and tetracycline. The study concluded that processed ready to eat meat and milk products available to consumers were contaminated with the virulent form of *L. monocytogenes* that is responsible for up to 95% of Listeriosis cases reported worldwide. It is recommended that strict regulation of the processing and storage conditions of ready to eat foods be done to ensure they reach consumers pathogen free

CHAPTER ONE : INTRODUCTION

1.1 Background information

Food borne diseases are an important public health concern worldwide and consists of a broad group of illnesses caused by a variety of aetiological agents including bacteria, viruses, parasites, fungi, chemicals, heavy metals and poisonous plants (Busani *et al.*, 2006; WHO, 2015; Addis and Sisay, 2015). The main mode of transmission of these diseases is through consumption of contaminated food and drinking water. Bacteria are by far the most commonly identified cause of food borne diseases with most of them being zoonotic in nature and having reservoirs in healthy food animals (Busani *et al.*, 2006; Raheem, 2016). Some of the most notable bacteria that cause food-borne diseases include *Staphylococcus aureus*, *Salmonella* species, *Campylobacter* species, *Listeria monocytogenes* and *Escherichia coli* (Abebe *et al*., 2020).

Listeriosis is a serious disease that is caused by *Listeria monocytogenes;* a bacterium that has been isolated from diversity of sources including soil, water, human and animal faeces and in a wide array of foods; meat, dairy products, eggs, vegetables, fish and other processed foods meant to be consumed without further heating or pasteurization (Schuppler and Loessner, 2010; Kuan *et al* 2013; Raheem, 2016). The natural habitat of the bacterium is thought to be decaying plant matter in which they live as saprophytes and faeces of many mammals (Vazquez-Boland, 2001; Seifi, 2012; Linke *et al.*, 2014). Listeriosis is reported to be the leading cause of death in reported cases of food borne infections, often having a mortality rate of between 30%-50% in some cases (Lindback *et al.*, 2011). The groups with the highest risk of

listeriosis are pregnant women, neonates, the elderly and the immunocompromised, where it manifests itself through septicaemia, meningitis, encephalitis, gastroenteritis and spontaneous abortions or still births in pregnant women (Liu, 2006; OIE, 2014). In animals, infection is mainly transmitted through the consumption of contaminated or spoilt silage in which *Listeria monocytogenes* readily multiplies leading to herd outbreaks (Vazquez-Boland, 2001).

Listeria monocytogenes is able to grow in varying conditions including gas or vacuum-packed products at refrigeration temperatures (Duffy *et al*., 1994), low water activity (Nolan *et al*., 1992) as well as low pH (Buchanan *et al*., 1993). It has also been reported to be resistant to cephalosporins and susceptible to a combination of ampicillin and an aminoglycoside (OIE, 2014). *Listeria monocytogenes* has been separated into at least thirteen serotypes that show varied virulence and pathogenicity (Liu *et al*., 2007; Liu, 2013). Serotypes 1/2a, 1/2b, 1/2c and 4b are responsible for 98% of outbreaks with serovar 4b being considered the most virulent (Jadhav *et al*., 2012). The largest listeriosis outbreak in history was reported in South Africa in 2018 with a mortality rate of 27%. In the outbreak, 42% of the cases were in neonates who were infected during pregnancy or delivery. Contaminated ready to eat meat, specifically polony was implicated as a major source of the outbreak. Other foods implicated were soft cheeses, unpasteurized milk and dairy products (National Listeria Incident Management Team, 2018; WHO, 2018). In June of 2019, six people died of a listeriosis outbreak in England attributed to pre-packaged sandwiches and salads that had been served in hospitals (Food Standards Agency report, 2019).

In Kenya, a study done by Njagi *et al.* (2004) in Nairobi and its environs on the presence of *Listeria* in indigenous chicken carcasses showed that *Listeria monocytogenes* was present in 22.2 % of collected samples. However, there is no published data on the occurrence of this pathogen in ready to eat foods of animal origin, specifically meat and milk products, in Kenya. The objective of the present study was therefore to determine the prevalence, molecular characteristics and antibiotic susceptibility of *Listeria monocytogenes* from milk, milk products and ready to eat meat products in Nairobi and its environs.

1.2 Statement of the problem

The prevalence of *Listeria monocytogenes* in foods, especially ready to eat foods of animal origin, has been reported in most developed countries (Mammina *et al*., 2009; Althaus *et al*., 2014; Miguel *et al*., 2015 and Quendera *et al*., 2016). However, in Kenya, no published study exists on the prevalence of this organism in processed ready to eat meat and milk products, the serotypes it exists in, the virulence factors these serotypes carry, the antibiotic susceptibility patterns and the genetic relationship of the organism to what has been reported elsewhere. This is despite a significant number of Kenyans being exposed to the risk factors of listeriosis namely; major changes in food production, increased use of refrigeration as a primary means of preservation of foods, increase in consumption of ready to eat foods mainly attributed to changing lifestyles and a growing middle class and an increase in the number of people considered at risk of the disease either due to age, cancer and immunosuppressive therapies, HIV/AIDS or pregnancy (Rocourt,1996; Evans and Redmond, 2013). Information is also not available on the antibiotic susceptibility

patterns of this organism in Kenya despite the reported rising level of antimicrobial resistance to commonly prescribed antibiotics.

1.3 Justification for the study

Research has shown that the key foodborne pathogens responsible for majority of foodborne illnesses internationally are *Campylobacter, Salmonella, Escherichia coli* O157 and *Listeria* (Evans and Redmond, 2013). Of these pathogens, *Listeria* is very important due to its ubiquitous nature, ability to persist in the food production environment, survive under refrigeration temperatures and causes listeriosis, a disease with a high fatality rate compared to diseases caused by other foodborne pathogens (Thakur *et al*., 2018).

Listeriosis is a major public health disease in many countries of the developed world but its representation in most developing countries especially in Africa is unclear due to limited research on *Listeria monocytogenes* (Odetokun and Adetunji, 2016). However, studies conducted in Ethiopia (Derra *et al.*, 2013; Seyoum *et al.*, 2015), Uganda (Mugampoza *et al.,* 2011), Botswana (Morobe *et al.,* 2009) Nigeria (Usman *et al.,* 2016) and Algeria (Bouayad and Hamdi, 2012) have been able to report the prevalence, virulence factors and serotypes of *Listeria monocytogenes* isolated from ready to eat foods. Currently in Kenya, there are no published studies on *L. monocytogenes* in ready to eat foods and there is therefore an urgent need to carry out a study to enable development of effective control measures.

The main mode of transmission of *L. monocytogenes* is through consumption of contaminated food of animal origin, especially ready to eat foods of animal origin, vegetables and contaminated water. The bacterium has the ability to survive in a wide range of environments, especially salty environment and refrigeration temperatures between 2°C-4°C. It has been reported that the number of *L. monocytogenes* cells can rise in refrigerated foods from fewer than 100 cells/ gram to more than 10,000 cells /gram (Huss *et al*, 2000; Mc Lauchlin *et al*., 2004), a very important factor in the storage of ready to eat foods. The infective dose of *L. monocytogenes* depends on the immune status of the host and it is estimated to be between 10^2-10^6 cells (Arun, 2008). This puts immunocompromised people such as those with AIDS, cancer, organ transplant recipients, and high-risk people such as the elderly, infants, neonates and pregnant women at risk of infection (Gilmartin *et al*., 2016).

According to the National Census conducted in 2019, the study area has a population of more than 7 million people with most of these people living and working within the major urban centres in this region (KNBS, 2019). Most of the shopping in urban centres takes place in retail markets due to the wide range of products that they offer including ready to eat foods. An increase in disposable income and changing lifestyles has led to an increase in consumption of ready to eat and ready to cook foods that entail minimal processing (Tchatchouang *et al*., 2020). Some of these ready to eat foods are stored under refrigerator conditions which provide an opportunity for *L. monocytogenes* to multiply to large numbers especially towards the end of the shelf life (Lopez-Valladares *et al.,* 2018) and may thus act as a source of infection. This

justifies the need to conduct a study on the prevalence of *Listeria monocytogenes* and its associated epidemiological factors in Nairobi and its environs.

Furthermore, with meat and milk being the food products commonly associated with contamination with *Listeria monocytogenes* (Raheem, 2016), it seems prudent to investigate samples from these main food products for this pathogen. In addition, with indiscriminate use of antibiotics by many people due to easy access of drugs over the counter coupled with frequent treatments of livestock with antibiotics and their use as growth promoters, any infecting bacterium would easily develop resistance to these drugs (Lungu *et al*., 2011; Wilson *et al*., 2018). A strategic disease control, drug change and or formulation must be based on sensitivity of the target pathogen to the drugs of choice and hence the need for evaluation of antibiotic resistance in *Listeria monocytogenes* isolates from the food samples in the present study.

1.4 Research questions

i) What is the prevalence of *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs?

ii) What are the major serotypes of the isolated *Listeria monocytogenes*?

iii) Which virulence factors do the *Listeria monocytogenes* isolates have?

iv) What are the genetic relationships between the *Listeria monocytogenes* isolates?

v) What are the antibiotic susceptibility patterns of the *Listeria monocytogenes* isolates?

1.5 Hypotheses

i) There's low prevalence of *Listeria monocytogenes* in ready to eat milk and meat products sold in retail markets in the study area.

ii) There are no major serotypes of *Listeria monocytogenes* in ready to eat milk and meat products sold in retail markets in the study area.

iii) There are no virulence factors present in *Listeria monocytogenes* from ready to eat milk and meat products sold in retail markets in the study area.

iv) There is no genetic relationship between *Listeria monocytogenes* isolates from ready to eat milk and meat products sold in retail markets in the study area.

v) *Listeria monocytogenes* from ready to eat milk and meat products is not susceptible to commonly used antibiotics in humans and animals.

1.6 Objectives

1.6.1 General Objective

To determine the prevalence, molecular characteristics and antibiotic susceptibility of *Listeria monocytogenes* from milk and meat products in Nairobi and its environs.

1.6.2 Specific Objectives

i) To determine the prevalence of *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs.

ii) To determine the major serotypes of *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs.

iii) To determine the virulence factors present in isolated *Listeria monocytogenes* from milk and meat products in retail markets in Nairobi and its environs.

iv) To determine the genetic relationship of the isolated *Listeria monocytogenes* from milk and meat products in retail markets in Nairobi and its environs.

v) To determine the antibiotic susceptibility patterns of *Listeria monocytogenes* isolated from milk and meat products in retail markets in Nairobi and its environs.

1.7 Significance and outcomes of the study

There is currently scanty scientific data on the prevalence of *Listeria monocytogenes* in both humans and animals in Kenya. This study aimed at providing clear scientific evidence on *Listeria* especially in relation to ready to eat foods of animal origin that are sold in retail markets where refrigeration is a common method of preservation. The study determined the prevalence, the most common serotypes, the virulence factors, the phylogenetic relationship and the antibiotic susceptibility of this particular microorganism. The results of this study will contribute significantly to the meat and dairy industry stakeholders, human and animal health practitioners, policy makers and the general public in that they will be better informed on the organism and will be able to make appropriate disease control decisions.

CHAPTER TWO : LITERATURE REVIEW

2.1 Food borne diseases

Food borne illnesses are diseases of infectious or toxic nature that result from the consumption of contaminated food or water (Adley and Ryan, 2016). These diseases have remained a global public health challenge worldwide due to a variety of factors including a growing population, immunocompromised people due to age and disease, international trade and travel which has led to the rapid distribution of foods worldwide and spread of pathogens to new geographical areas, evolution of microorganisms that are resistant to drugs used against them, exposure to unfamiliar food borne hazards through consumption of foods prepared outside the home and the breakdown of public health measures (WHO, 2015; Adley and Ryan, 2016; Feltes *et al*., 2017).

There are about 31 pathogens known to cause a majority of the food borne illnesses of which 21 are bacteria such as *Bacillus cereus*, *Brucella* species and *Campylobacter jejuni*, 5 parasites such as *Cryptosporidium* species and *Trichinella* species and 5 viruses such as Rota viruses, and Noro viruses (Adley and Ryan, 2016). In a report by WHO (2015), these pathogens were reported to have caused 600 million food borne illness and 420,000 deaths in 2010 including 125,000 children under the age of five years. Half of the global burden of food borne diseases is caused by diarrhoeal diseases affecting 550 million people per year and causing 230,000 deaths every year especially in children. The main cause of these diarrhoeal diseases is eating contaminated raw or undercooked meat, eggs, fresh produce and dairy products (WHO, 2015). Bacteria are the most common causes of food borne disease outbreaks.

The bacteria involved include *Salmonella spp, Bacillus cereus, Campylobacter spp,* Shiga toxin-producing *Escherichia coli* (*E. coli*) and *Listeria monocytogenes* (Altekruse *et al*., 1997; Heredia and Garcia, 2018). Of these bacterial pathogens *L. monocytogenes* is reported to be highly implicated in food contamination leading to frequent food borne outbreaks in diverse regions of the world.

2.1.1 Listeriosis

Listeriosis, caused by *Listeria monocytogenes*, is an important food borne disease that can be difficult to control and commonly results in severe clinical outcomes. The disease can occur sporadically or in an outbreak (OIE, 2014). It is a zoonotic disease that affects a wide variety of domestic animals, wild animals, birds and fish which carry *Listeria monocytogenes* in their digestive systems. Domestic animals may acquire the bacterium from contaminated feed and water, inhalation or venereal transmission and may shed it in faeces, milk, uterine discharges, nasal discharges and urine. It may manifest as abortions, encephalitis or meningoencephalitis, septicaemia and myocardial necrosis (CFSPH, 2005; MVM, 2016).

Humans get infected by ingestion of a wide range of contaminated foods such as meat products, dairy products especially soft cheese, salad vegetables, fish and sea food products, delicatessen products and industrially produced refrigerated ready to eat foods that don’t require further cooking or reheating (Vazquez-Boland *et al*, 2001; Ponniah *et al.*, 2010; CDC, 2011; OIE, 2014). The bacterium has been associated with a mortality rate of between 30-50% (Lindback *et al*, 2011) with the highest risk groups being pregnant women, neonates, the elderly and the immunocompromised. In

the early stages of infection, non-specific flu like symptoms; chills, fatigue, headaches, muscular and joint pains accompanied by gastroenteritis are common. Without proper treatment, the infection can develop into septicaemia, meningitis, encephalitis, spontaneous abortions or still births and in some cases death (Vazquez-Boland *et al*, 2001; Liu, 2006; OIE, 2014).

2.2 The biology of Listeria monocytogenes

Listeria monocytogenes belongs to the genus Listeria; rod shaped bacteria of 0.4-0.5µm by 1-1.5µm in size, gram positive, motile at 10˚C to 25˚C, facultative anaerobic, non-spore forming and found in a wide range of environments including water, soil, effluent, and a variety of foods (Vazquez-Boland *et al*, 2001; Liu, 2006; Liu, 2013). The genus was first described by Murray *et al*. (1926) when it was isolated as the etiological agent of a septicaemic disease that was affecting rabbits and guinea pigs in the laboratory.

The genus is classified into family *Listeriaceae,* order *Bacillales*, class *Bacilli*, phylum *Furmicutes*, domain *Bacteria* and kingdom *Prokaryotae* (Liu, 2013). The other genus in the family is Brochothrix, which has two species namely *Brochothrix thermosphacta* and *Brochothrix campestris*. Bacteria in the genus Listeria are closely related to Bacillus, Clostridium, Enterococcus, Streptococcus and Staphylococcus (Vazquez-Boland *et al*, 2001). Recent classification has grouped the genus Listeria into 18 species organized into 2 major groups. The first group, referred to as *"Listeria sensu stricto",* is composed of six species namely; *L. monocytogenes, L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. marthii.* These species have mainly been

isolated from the intestinal tract of symptomless animals and foods of animal origin (Schardt *et al*, 2017). The second group referred to as *"Listeria sensu lato"* is composed of the other twelve species namely; *L. grayi, L. rocourtiae, L. fleischmannii, L. weihenstephanensis, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. grandensis, L. riparia, L. costaricensis* and *L. booriae.* These species have been isolated from food associated surfaces and the environment. They are however unable to colonize mammalian hosts (Schardt *et al*, 2017). Only *L. monocytogenes* and *L. ivanovii* have been found to be pathogenic (Robinson *et al*, 2000; Liu, 2013; Orsi and Wiedmann, 2016).

Organisms in the genus Listeria are tolerant to pH changes as low as 4.4, temperature, high salinity (40% w/v), low water content and hypoxic conditions (Liu *et al.*, 2005; Meloni *et al* 2009; Al-Nabulsi *et al* 2015). *Listeria monocytogenes* is able to employ several strategies to evade the immune system and thus promote its survival. First, the organism is able to create an intracellular niche through its capacity to adhere, invade and multiply within non-phagocytic cells (Cossart and Lecuit, 1998). This leads to its spread without being exposed to antibodies, neutrophils, or antibiotics in the extracellular fluid (Hof *et al.*, 1997). The organism is also able to modify bacterial ligands for pattern recognition receptors in innate immunity, modulate host signalling pathways and target host immune effector cells. Cell mediated immunity therefore becomes the hosts' only defence mechanism against this organism and any condition that reduces this type of immunity, for instance, pregnancy or chronic illnesses can predispose to listerial infection. This may explain how the organism is able to cross

the placental barrier or blood brain barrier and cause abortions and meningitis respectively (Temple and Nahata, 2000; Janakiraman, 2008).

2.3 *Listeria monocytogenes* serotypes and lineages

The genus Listeria is divided into at least 16 serotypes based on the immunological reaction between the somatic O antigen, the flagella H antigens and specific antibodies. *Listeria monocytogenes* has 13 of the 16 serotypes which are; 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Virulence is serotype dependent with serotypes 1/2a, 1/2b, 1/2c and 4b being involved in 98% of human cases (Chen and Knabel, 2007; Liu, 2013). Serotypes 1/2a, 1/2b and 1/2c are mainly implicated in sporadic listeriosis while serotype 4b is implicated in endemic listeriosis in humans.

Due to extensive antigenic sharing, serotyping lacks the desired specificity hence through genotyping, the serotypes can be further grouped into four lineages, I, II, III and IV. Lineage I consist of serotype 1/2b, 3b, 4b, 4d and 4e. Lineage II consists of serotypes 1/2a, 1/2c, 3a, and 3c while lineage III comprises serotypes 4a and 4c and Lineage IV consists of non-serovar 4a, non-serovar 4c and 7 (Chen and Knabel, 2007; Ward *et al.*, 2008). Lineage I has been associated with a majority of human listeriosis cases, lineage II has been associated with food and environmental sources, while lineage III has been associated with animal hosts as shown in Table 2.1.

Table 2.1: *Listeria monocytogenes* lineages, serotypes and potential sources (Kathariou, 2002)

Lineage	Serotypes	Potential Source
I	1/2b 3b 4b 4d 4e	Human Listeriosis cases
II	1/2a 1/2c 3a 3c	Food and environmental sources, animal listeriosis cases and sporadic human clinical cases
III	4a 4c	Animal sources
III c	4c	Animal sources
IV	7 and unusual 4a, 4b and 4c	Animal sources

2.4 Virulence factors and pathogenesis of *Listeria monocytogenes*

The ability of *L. monocytogenes* to cause disease correlates with its capacity to survive within macrophages, to invade non-phagocytic cells, replicate and cross the intestinal, blood-brain and foetal placental barriers (Lecuit, 2005; Cossart, 2011; Pizarro-Cerda *et al*, 2012). *Listeria monocytogenes* enters the susceptible host primarily through ingestion of contaminated food and is able to withstand the acidic environment of the stomach, proteolytic enzymes of the host, bile salts and non-specific inflammatory attacks mainly through the help of stress response genes (Sleator *et al.,* 2003). It then adheres to, is internalized and survives in host cells aided by a host of virulence factors that are encoded in a cluster of six genes which are 9 kb

in length collectively known as the virulence gene cluster or Listeria Pathogenicity Island 1. This cluster of genes is present in three species of Listeria namely *L. monocytogenes*, *L. ivanovii* and the non-pathogenic *L. seeligeri* which has an additional five genes in its gene cluster (Schmid *et al.*, 2005). The virulence gene cluster in *L. monocytogenes* codes for a variety of virulence factors which include Internalin A, Internalin B, Listeriolysin O, Phospolipases, surface protein ActA and Metalloproteases.

Internalin A is a listerial surface protein that is involved in the penetration of the bacterium in non-phagocytic cells such as epithelial cells by binding to a surface protein, E- cadherin, on the surface of the host cell in a process that promotes phagocytosis. E- cadherin is involved in the formation of adherens junctions at the intestinal barrier, blood brain barrier and the placenta. Internalin B interacts with the hepatocyte growth factor receptor c-Met to promote internalization in a wide variety of mammalian cells. It has been implicated in colonization of the liver after intravenous infection in mice (Pentecost *et al*, 2010; Pizarro-Cerda *et al*, 2012). After *Listeria monocytogenes* is engulfed in a phagocytic vacuole, it ensures its viability by preventing phagosome maturation to the phagolysosomal stage (Vazquez-Boland, 2001). Listeriolysin O (LLO) is a virulence factor that is encoded by the gene *hly* and is involved in the escape of *L. monocytogenes* from phagosomes of the host cell. It does this through lyses of the phagosome membrane leading to the escape of the *Listeria monocytogenes* to the cytoplasm of the cell. The LLO is later recognized by enzymes in the cytoplasm of the cells and is destroyed before it can damage the cell membrane (Doyle, 2001).

Once the *Listeria monocytogenes* is released into the cytoplasm, it replicates before embarking on the process of intracellular spread. This process is aided by another virulence factor, ActA, which leads to polymerization of actin molecules to form actin filaments. The filaments form an actin tail that is up to 40μm at one of the bacterial poles which propels the bacterium within the cytoplasm until it reaches the cell periphery and comes into contact with the cell membrane where it causes portions of the membrane to bulge outwards forming structures referred to as listeriopods (Vazquez-Boland, 2001; Doyle, 2001). These protuberances are engulfed by adjacent cells, thereby allowing dissemination of *Listeria monocytogenes* without exposure to antibodies or other immunoactive molecules (Doyle, 2001).

Two phospholipases, phosphatidylinositol specific phospholipase C and phosphatidylcholine specific phospholipase C also facilitate the lysing of the host cell membranes. Phosphatidylinositol specific phospholipase C (PI-PLC) aids in the escape of *Listeria monocytogenes* from the primary vacuole on initial infection while phosphatidylcholine specific phospholipase C (PC-PLC) aids in the escape of *Listeria monocytogenes* after cell to cell spread (Doyle, 2001) (Figure 2.1). A bacterial zinc dependent metalloprotease and a host cell cysteine protease is required to cleave off part of the precursor and activate PC-PLC whereas PI-PLC is synthesized in an active form.

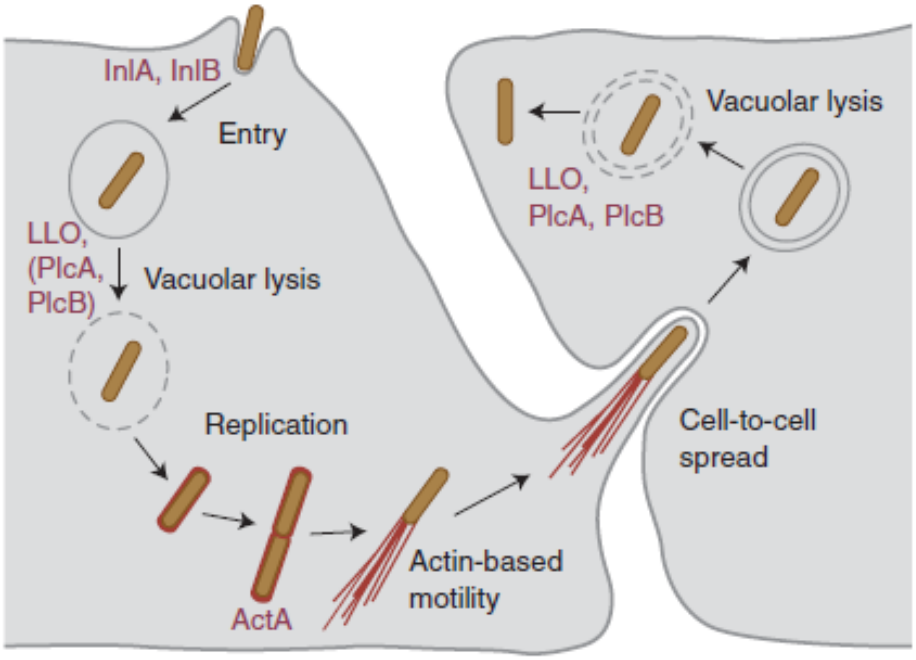

Figure 2.1: Intracellular cell cycle of *Listeria monocytogenes* (Pizarro-Cerdá *et al*., 2012)

2.5 Epidemiology of listeriosis

Listeria was first described by Murray in 1926 as the causative agent of monocytosis in laboratory rodents. However, it was not until the late 70's to early 80's that the link between the organism and serious food borne listeriosis was made (Schlech *et al.*, 1983; Mc Carthy, 1990). Since then, several cases of the disease and isolation of the causative agent from clinical samples and foods have been reported worldwide, mostly in developed countries where incidences are low but mortality high. Some of the reported cases are in Israel (Yardena *et al.*, 2002), Italy (Mammina *et al.*, 2009), the Czech republic (Gelbicova and Karpiskova, 2009), Switzerland (Althaus *et al.*, 2014), Serbia (Gusman *et al*., 2014), Spain (Miguel *et al.*, 2015), Turkey (Terzi *et al*., 2015), Iran (Maktabi *et al.*, 2015), Portugal (Quendera *et al.*, 2016), India (Barbuddhe

et al., 2012), Brazil (Abrahão *et al.*, 2008), China (Wu *et al.*, 2015), Malaysia (Kuan *et al.*, 2013) and the United States of America (CDC, 2011). Nine hospital patients died in an outbreak reported in June 2019 in the United Kingdom. The outbreak was linked to pre-packaged sandwiches that were served to the patients (Food Standards Agency, United Kingdom, 2019).

Cases involving Listeria in developing countries are rarely reported and this may be attributed to ineffective control or surveillance strategies (Raheem, 2016). However, studies have been carried out in several African countries on the prevalence of *L. monocytogenes* in foods. In Nigeria, Usman *et al.* (2016) characterized the virulence genes using PCR of 36 isolates from raw milk and milk products. Eruteya and Odunfa (2014) were able to recover four strains from goat meat and isolated virulence genes using PCR, while Nwaiwu (2016) was able to determine the molecular serotypes and evolutionary lineage of three samples isolated from various foods. In Egypt, the bacterium was isolated from beef luncheon, chicken luncheon, frankfurter beef and human stool samples (Awadallah and Suelam, 2014), while in Botswana, 57 (4.3%), out of 1,324 food samples collected were positive for *L. monocytogenes* with the bacterium mainly being isolated from frozen cabbage, coleslaw and cheese (Morobe *et al.*, 2009). The largest reported outbreak was in South Africa in 2018 where more than 1000 people were infected with listeriosis resulting in 674 hospitalizations and 216 deaths (National Listeria Incident Management Team, 2018; Tchatchouang *et al*., 2020).

In the Eastern Africa region, most of the published reports are from Ethiopia where Garedew *et al*. (2015) reported that 6.25% of ready-to-eat food samples were positive for *Listeria monocytogenes.* Seyoum *et al*. (2015), Derra *et al*. (2013) and Molla *et al*. (2004) carried out studies in Addis Ababa and surrounding regions in meat, milk and its products, and collectively showed the presence of the bacterium with a prevalence ranging between 4%-10%. In Uganda, Mugampoza *et al.* (2011) carried out research in bulked raw milk and traditionally fermented dairy products and reported a prevalence of 13% in bulked raw milk and 3% in locally processed yoghurt. In Kenya, Njagi *et al.* (2004) reported a prevalence of 22.2% in slaughtered indigenous chicken from Nairobi and its environs. Despite this high prevalence rate of *L. monocytoge*nes in foods, there is currently no officially published report on the prevalence of *L. monocytogenes* in ready to eat foods of animal origin in Kenya.

2.6 Diagnosis and treatment of listeriosis

The most common method of diagnosis of listeriosis is culturing the organism from a sterile site such as blood, amniotic fluid or the spinal fluid. Gram staining is useful only in a third of the cases and hence not reliable. This is because the organism is an intracellular bacterium and can entirely be missed or due to the organism resembling pneumocci, diphtheroids or haemophilus (Southwick and Purich, 1996). Monnier *et al.* (2011) have also reported on the diagnosis of *L. monocytogenes* meningoencephalitis using real time PCR for the *hly* gene.

The intracellular nature of *Listeria monocytogenes* makes its effective antimicrobial treatment difficult (Temple and Nahata, 2000; Hof, 2003; Pagliano *et al*., 2017). An

ideal antibiotic must have the ability to penetrate the host cell, bind tightly to an intracellular target and have the capacity to create depots therefore ensuring a long lasting optimal antibiotic concentration (Temple and Nahata, 2000). Organisms in the genus *Listeria* possess five penicillin binding proteins of which only penicillin binding protein 3 (PBP3) is identical in all *Listeria* species. An ideal antibiotic must also therefore have the capacity to bind to PBP3, which is involved in the late stages of peptidoglycan synthesis, a main component of the bacterial cell wall, and when blocked, leads to cell death (Hof *et al*., 1997). Based on these criteria, a variety of antibiotics are used, the most preferred being penicillin, amoxicillin and ampicillin (Hof, 2003).

It has been suggested that a combination of these drugs with gentamicin has a synergistic effect although animal models do not reliably show this (Temple and Nahata, 2000). For individuals who may be allergic to penicillins, the second line of treatment involves the use of trimethoprim/ sulfamethoxazole though it should be avoided in early pregnancy to prevent neural tube defects. Other antibiotics recommended include erythromycin, vancomycin and fluoroquinolones. Cephalosporins have no activity against PBP3 and therefore not active against *Listeria* (Temple and Nahata, 2000; Hof, 2003). The emergence of *Listeria monocytogenes* strains that are resistant to penicillin has been reported (Abdollahzadeh *et al*., 2016; Kuan *et al*., 2017). This resistance has mainly been attributed to the overuse or indiscriminate use of penicillin in both animals and humans (Kuan *et al*., 2017).

2.7 Isolation of *Listeria monocytogenes* from contaminated foodstuff

Listeria monocytogenes can be isolated from food, environmental and clinical samples using a variety of methods and culture media. The isolation methods must be able to detect 1 *Listeria* organism per 25 grams of food (Law *et al*., 2015). For this to happen, enrichment methods are required in order for the organisms to grow and reach a detection level of 10^4-10^5 cfu/ml before plating on selective media (Law *et al*., 2015). Selective and enrichment media contain antibiotics to suppress competing microflora since *Listeria* cells are slow growing and can be rapidly outgrown by competitors. Some of the most common agents include; acriflavine which inhibits growth of other gram-positive bacteria, nalidixic acid which inhibits growth of gram-negative bacteria and cycloheximide which inhibits growth of fungi (Jeyaletchumi *et al.*, 2010). Esculin, a carbohydrate, is also an essential component in *Listeria* enrichment and plating media. All *Listeria* species are capable of hydrolysing esculin and in the process leads to the formation of intense black colour in the media (Jeyaletchumi *et al.*, 2010).

Some of the most common methods applied in the isolation and detection of *Listeria* include: The Food and Drug Administration (FDA) / Bacteriological Analytical Manual (BAM) method, ISO11290 method and the United States Department of Agriculture (USDA) / association of analytical chemist method. These methods mainly differ on the choice of enrichment and selective media (Law *et al*., 2015).

2.7.1 Food and Drug Administration (FDA) method

This method is also referred to as the Bacteriological Analytical Manual (BAM) Method (2011). This method uses 25 grams of the food sample which is pre enriched in Listeria Enrichment Broth containing trypticase soy broth, yeast extract, acriflavine HCl, nalidixic acid, cycloheximide, and distilled or deionized water. The food sample is incubated at 30^{o}C for four hours after which selective agents (Acriflavin 10mg/l, Sodium nalixidate 40mg/l and optional antifungal 50mg/l) are added. Incubation continues at 30^{o}C for a total of 48 hours. Presumptive *Listeria* colonies which appear brownish black are then selected for purification in selective agar which can either be Oxford, PALCAM, MOX, or LPM (BAM, 2011).

2.7.2 International Organization for Standardization (ISO) 11290 method

This method involves enriching the food sample in Fraser broth, containing; proteose peptone, tryptone, yeast extract, sodium chloride, di-sodium hydrogen phosphate, potassium dihydrogen phosphate, aesculin and lithium chloride, in two consecutive stages. In the first stage, the food sample is enriched by culturing it in half Fraser broth, which contains nalidixic acid and acriflavine HCl in addition to the contents of Fraser broth, for 24 hours. In the second stage, an aliquot of the first enrichment is transferred into full strength Fraser broth for another 24 hours for further enrichment. Fraser broth has agents which are selective and only allow for the detection of β D glucosidase activity by Listeria. Presence of organisms in the genus Listeria in the test sample leads to the blackening of the medium. A loopful of the primary and

secondary enrichment broth is then plated on Oxford and PALCAM agars (Gasanov *et al.,* 2005).

2.7.3 United States Department of Agriculture (USDA) Method

This method is also referred to as the Association of Analytical Chemists Method. It is a method of choice for isolation and analysis for meat, eggs, poultry and environmental samples. It is a two-stage enrichment process that involves primary enrichment of the food sample using the University of Vermont medium which contains; proteose peptone, yeast extract, beef extract, sodium chloride, di-sodium hydrogen phosphate, potassium dihydrogen phosphate, aesculin, nalidixic acid and acriflavine hydrochloride and secondary enrichment in Fraser Broth. Plating of the enriched sample is carried out in modified Oxford agar containing selective agents (Gasanov *et al.*, 2005).

2.8 Identification of isolated *Listeria* species

Identification of Listeria uses phenotypic and biochemical markers; where the bacterium is differentiated from other bacteria by the esculinase reaction which is based on β-D-glucosidase activity. The *Listeria* colonies appear black with a black zone in surrounding medium. The bacterium is gram positive on gram staining technique, aerobic and facultative anaerobic, non-spore forming, catalase positive, oxidase negative, ferments sugars without gas and are motile at 28^{o}C (Gasanov *et al.*, 2005).

2.8.1 Christie, Atkins, Munch- Petersen (CAMP) test

The CAMP test is used to differentiate between the haemolytic species in the genus *Listeria* namely; *Listeria monocytogenes*, *Listeria ivanovii* and *L. seeligeri*. It is done by streaking β hemolysin producing *Staphylococcus aureus* and *Rhodococcus equi* parallel to each other on 5% sheep blood agar followed by streaking a colony of the suspect *Listeria* cultures at right angles in between the parallel streaks. The plate is then incubated for 18-24 hours at 37°C. Hemolysis by *Listeria ivanovii* is enhanced within the vicinity of *Rhodococcus equi* while that of *L. monocytogenes* and *L. seeligeri* is enhanced in the vicinity of *S. aureus* (Robinson *et al.*, 2000; Gasanov *et al.*, 2005).

2.8.2 Biochemical tests

Biochemical tests employed for the identification of organisms in the genus *Listeria* include the catalase test, oxidase test, methyl red, Voges-Proskauer, nitrate and sugar fermentation tests with xylose, rhamnose, mannitol and α-methyl D mannopyranoside. The catalase test is based on the neutralization of hydrogen peroxide by microorganisms that produce the enzyme catalase which mediates the breakdown of hydrogen peroxide into water and oxygen gas. This enzyme is mainly produced by aerobic bacteria and some facultative anaerobes. The oxidase test is based on the principle that certain bacteria possess either cytochrome oxidase or indophenol oxidase. These compounds catalyse the transport of electrons from donor compounds to electron acceptors. The test involves the incorporation of an artificial electron acceptor such as N, N-dimethyl-p-phenylenediamine, a colourless dye, in discs. When oxidase producing organisms are introduced on the discs, the reaction

between the enzyme and the artificial electron acceptor leads to the production of a blue colour on the discs. The methyl red test is based on the principle that some bacteria are able to utilize glucose and convert it to stable acids such as lactic acid, acetic acid or formic acid. The acid produced changes the pH of the broth containing the test organism to below 4.5. Addition of methyl red to this broth leads to change of colour from yellow to red. The Voges-Proskauer test is used to determine whether an organism produces acetylmethyl carbinol from glucose fermentation. If the compound is present, it is converted to diacetyl in the presence of α-naphthol, 40% potassium hydroxide and oxygen. A positive test leads to the formation of a red-pink solution (BAM, 2011; Hemraj *et al*, 2013).

The sugar or carbohydrate fermentation tests are used to determine whether or not a bacterium can ferment a particular sugar. A pH indicator is incorporated in the medium to determine whether or not acid is produced (BAM, 2011). The expected results for the common *Listeria* species to these biochemical tests is as indicated in Table 2.2.

Table 2.2: Biochemical tests and expected results in the identification of *Listeria* species (Gasanov *et al*, 2005)

	L. monocytogenes	***L. innocua***	***L. ivanovii***	***L. seeligeri***	***L. welshimeri***	***L. grayi***
Hemolysin	+	-	+	+	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-
L-Rhamnose	+	+/-	-	-	+/-	+/-
D- Mannitol	-	-	-	-	-	+
D-Xylose	-	-	+	+	+	-
α-methyl mannoside	+	+	-	-	+	+

2.8.3 Chromogenic substrates

In the culture and isolation of *Listeria* species using PALCAM and Oxford agar, a limitation was faced in the differentiation of pathogenic from non-pathogenic *Listeria* (Law *et al*., 2015). This led to the development of chromogenic substrates which were incorporated in plating media and led to the detection of essential determinants of pathogenicity and hence the ability to differentiate between pathogenic and non-pathogenic *Listeria* by production of characteristic colour (Hitchins, 2003; Gasanov *et al.*, 2005; Law *et al*., 2015). *Listeria monocytogenes* and *Listeria ivanovii* produce phosphatidylinositol specific phospholipase C (PIPL-C) enzyme whose activity can be detected by chromogenic media (blue colonies) (Coffey *et al.*, 1996).

2.8.4 Antibody based tests for detection of *Listeria* species

These are simple, sensitive and accurate tests that are based on the detection of antibodies specific to *Listeria*. They are mainly applied in food testing and can be carried out after enrichment. These tests include: the enzyme linked immunosorbent assay (ELISA) and immunocapture. In the ELISA test, antibodies are immobilized in microtitre wells for antigen capture in combination with a secondary antibody attached to an enzyme to detect the captured antigen (Gasanov *et al.*, 2005). In the immunocapture test, magnetic beads or dip sticks that are coated with *Listeria* specific antibodies are used to separate the organism from competing microflora and inhibitory food components in a sample (Gasanov *et al.*, 2005).

2.8.5 Molecular methods for the detection of *Listeria* species

Molecular methods are extremely accurate, sensitive and specific. They include DNA hybridization and polymerase chain reaction. DNA hybridization is a method used in the detection of specific microorganisms e.g. Listeria species in food using a target DNA sequence. The method involves the use of a probe made of complimentary sequence to the target DNA sequence in the microorganism of interest. The probe contains a radioactive or fluorescent label to indicate detection (Jeyaletchumi *et al.*, 2010). Polymerase chain reaction is a technique where sections of DNA are amplified using heat stable primers and DNA polymerase. The amplified segments are then detected using agarose gel electrophoresis. The test is able to amplify large amounts of DNA from minute amounts of target DNA. Multiplex PCR, is a type of PCR where multiple primer sets are used. This allows for simultaneous detection of more than one pathogen in the same sample (Hudson *et al.*, 2001).

2.8.6 Typing of Listeria monocytogenes

Typing is used to discern closely related *Listeria monocytogenes* strains through several methods which include: serological typing, phage typing, multilocus enzyme electrophoresis and molecular methods. Serological typing is based on the detection of somatic ('O' antigens) and flagellar ('H') antigens in *Listeria* species through the use of antibodies that specifically react with these antigens. This then enables the classification of *Listeria* into serotypes based on which of these antigens is present. It can also be done using commercial kits (Jeyaletchumi *et al.*, 2010). Phage typing is based on the interaction of certain bacteriophages with their *Listeria* host strain resulting in cell lysis. Not all *Listeria monocytogenes* are typable especially serotype 1/2 strains (Gasanov *et al.*, 2005; Jeyaletchumi *et al.*, 2010).

Multilocus enzyme electrophoresis is based on variations in the amino acid sequence of enzymes which results in a different electrostatic charge of the protein and different electrophoretic mobility. The difference in mobility is directly related to allelic variations in gene coding of these enzymes (Gasanov *et al.*, 2005). Ribotyping is based on the detection of variations in ribosomal proteins or genes. The most useful gene for evaluation of phylogenetic relationship is the gene coding for ribosomal RNA. It involves ribotyping *Listeria* isolates where restriction enzymes are used to digest chromosomal DNA. This is followed by DNA hybridization using ribosomal RNA gene probes. *Listeria* isolates are sorted into ribotypes and their relatedness established by the banding patterns that result. (Gasanov *et al.*, 2005; Jeyaletchumi *et al.*, 2010).

Restriction enzyme analysis involves the use of restriction enzymes which detect and digest particular sequences within a DNA molecule. This leads to production of varying DNA fragments whose genetic relatedness can be determined by comparing the number and size of the fragments, and separating and visualizing them through gel electrophoresis (Liu, 2006). PCR typing is the amplification of specific target sequences and analysing their PCR products or their restriction enzyme digests by comparing lengths of DNA fragments. It is carried out through several methods which include the Random Amplification of Polymorphic DNA (RAPD) and arbitrary primed PCR, amplified fragments length polymorphism, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and repetitive extragenic palindrome-polymerase chain reaction (REP-PCR) (Jeyaletchumi *et al.*, 2010). Target genes include internalin genes (*inlA, inlC,* and *inlJ*), Listeriolysin O gene (*hlyA*), Actin gene (*act A*) and phosphatidyl-inositol-phospholipase C gene (*plcA*) (Liu *et al.*, 2007).

2.9 DNA sequencing

Deoxyribonucleic acid is the blue print of life that is made up of building blocks referred to as nucleotides. These nucleotides are composed of a phosphate group, a sugar group and one of four nitrogenous bases namely; Adenine, Cytosine, Guanine and Thymine. Nucleotides link together to form a strand of DNA and depending on the order of the bases, the instructions in a DNA strand can be deciphered (Alberts *et al,* 2002). DNA sequencing is therefore a method of determining the order of the nucleotide bases in a strand of DNA (Kumar, 2012). This knowledge is beneficial in basic biological research, forensics and diagnostics. There are several sequencing

methods that have been developed since the early seventies with the most notable being the Maxam-Gilbert method, Chain termination method or Sanger sequencing and the Next generation sequencing methods (Kumar, 2012).

2.9.1 Maxam-Gilbert method

This method of DNA sequencing is based on chemical alteration of the DNA molecule followed by cleavage at specific bases. It involves the use of piperidine which catalyzes the cleavage of phosphodiester bonds, dimethyl sulfate which reacts with purines and hydrazine which reacts with pyrimidines. This leads to the generation of small fragments which are then separated by gel electrophoresis according to size (Kumar, 2012).

2.9.2 Chain termination /Sanger method

In this method, the target DNA is copied many times making fragments of different lengths. The ends of these fragments are marked by dideoxynucleotides which are similar to regular nucleotides apart from the lack of a hydroxyl group on the 3' carbon of the sugar ring. This hydroxyl group when present is responsible for addition of nucleotides to an existing chain. Sanger sequencing begins with denaturation of the DNA double strand. This is then followed by addition of primers, DNA polymerase, DNA nucleotides and dideoxynucleotides although in a smaller amount than the ordinary nucleotides. The dideoxynucleotides have a fluorescent marker attached which allows fluorescence based on the associated nucleotide (Kumar, 2012; Gomes and Korf, 2018).

2.9.3 Next generation sequencing

This is a term that is used to describe several modern sequencing technologies that have been developed from the early years of the 21st century. These technologies are able to sequence DNA and RNA much faster and at a lower cost than Sanger and Maxam- Gilbert sequencing. They also do not require PCR amplification and electrophoresis since the sequencing output is detected in real time. Some of these technologies include; Pacific Biosciences SMRT sequencing, Oxford nanopore sequencing, Illumina /Solexa sequencing and ion torrent sequencing (Kchouk *et al*, 2017).

2.10 Phylogenetic analysis

Phylogenetic analysis is a method of determining the evolutionary history and relatedness of a group of organisms (Horiike, 2016). Initially, phylogenetic analysis was done by morphological comparisons of organisms but currently, it has developed to include the study of DNA sequences of genes, RNA sequences or amino acid sequences of proteins and trying to deduce their evolution by comparing them with homologues. The knowledge that is obtained contributes to both basic and applied biology including research into diseases and the development of vaccines (Lam *et al*, 2010; Horiike, 2016). In order for precise information on sequence similarity to be obtained, the sequence under study has to be compared with other sequences stored in data bases. This comparison is referred to as a homology search and is carried out by homology search programs such as the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*, 1997).

Once homology is confirmed, Multiple Sequence Alignment (MSA) is carried out where three or more biological sequences of DNA, protein or RNA are available. Alignment is used to calculate the best match for selected sequences and identify similarities and differences (Horiike, 2016). On completion of alignment, inference on the aligned sequences into a phylogenetic tree is made using either distance-based methods or character-based methods. Distance based methods include the Unweighted Pair Group Method with Arithmetic (UPGMA) and the Neighbour- Joining method (NJ). Character based methods include the Maximum Parsimony, Maximum Likelihood and Bayes method. The reliability of the tree is determined by the tree shape, the branch lengths and performing bootstrap test (Horiike, 2016).

2.11 Antibiotic resistance in *L. monocytogenes* isolates

Listeria monocytogenes is susceptible to a wide range of antibiotics, but is also resistant to others. In a study done in Malaysia by Kuan *et al.* (2017), *Listeria monocytogenes* isolates were found to have been resistant to penicillin G, rifampicin and meropenem while being susceptible to gentamicin, ampicillin, and trimethoprim sulfamethoxazole. In another study by Ruiz-Bolivar *et al.* (2011) in Colombia, the organism was found to be highly susceptible to ampicillin, amoxicillin/clavulanic acid and chloramphenicol. In Botswana, Morobe *et al.* (2009) found 54% of *Listeria monocytogenes* isolates to be resistant to one or more antibiotics. Antibiotic resistance is mainly attributed to resistance genes namely; *erm(A), erm(B), erm(C), erm(TR), mef(A)* and *msr(A)* for macrolide-lincosamide-streptogramin B, *dfrD* for trimethoprim, *tet(M), tet (S), tet(K)* and *tet (L)* for tetracyclines (Morvan *et al*., 2010). The first multidrug resistant Listeria isolate was identified in France in 1988 and since

then, many more resistant strains have been isolated from food (Poyart-Salmeron *et al.*, 1990; Rota *et al.*, 1996; Walsh *et al.*, 2001). The isolates acquire resistance through a number of mechanisms such as acquiring mobile genetic elements namely; self-transferable, mobilizable plasmids and conjugative transposons and target gene mutations such as mutation within genes encoding efflux pumps (Luque-Sastre *et al.*, 2018).

Testing for antibiotic sensitivity and resistance can be carried out through a variety of methods, among them the Kirby Bauer disc diffusion method and the Epsilometer test. In the Kirby Bauer test, the test microorganism is spread on Muller Hinton agar and let to grow. Antibiotic impregnated discs are later introduced and the plates incubated at 37 $^{\circ}$C for 24 hours after which any zones of inhibition are measured and susceptible, intermediate and resistant categories assigned (Hudzicki, 2009). The Epsilometer is a method of determining antimicrobial resistance where a rectangular plastic test strip with predefined, continuous and exponential gradient of antibiotic concentration is placed on a plate with Muller Hinton agar and the test organism and incubated for 48 hours after which a symmetrical inhibition ellipse is produced. The intersection of the zone of inhibition and the calibrated antibiotic strip indicates the minimum inhibitory concentration (Acharya, 2015).

2.12 Future perspective

Based on the literature review, it is clear that *Listeria monocytogenes* is prevalent in foods especially ready to eat foods that do not require heat treatment before consumption. The presence of this organism in these foods may be due to contamination during processing or post processing. There is therefore an urgent need to audit some of the products which are sold as ready to eat for the presence of this organism, especially so in a city like Nairobi and its environs where more than 6.5 million people reside. These people are reliant in one way or another on products that are sold in retail markets and that have been identified as potential sources of listeriosis. This coupled with an increasing population of people at risk that is young, elderly and immunocompromised and recent outbreaks in South Africa and England makes it necessary for a study to be conducted not only in foods but also in humans and animals to determine the major epidemic clones in circulation so that we are better prepared in case an outbreak occurs.

Listeriosis is managed through use of antimicrobials, mainly penicillin in combination with gentamicin. However, resistance to antimicrobials used in humans and animals has been an emerging issue worldwide. This has been mainly attributed to overuse of antimicrobial agents in animal and human health for prophylaxis and treatment, widespread availability of counterfeit or substandard drugs and poor infection control measures. With listeriosis being an important food borne disease especially, it is necessary to also continuously monitor *Listeria monocytogenes* isolates from such foods, the environment, humans and animals to determine possible mechanism of drug resistance, and adopt appropriate preventive measures.

CHAPTER THREE : MATERIALS AND METHODS

3.1 Study area

The study was conducted in selected commercial centres in Nairobi County and in the surrounding counties of Kiambu, Kajiado and Machakos, an area collectively referred to as the greater Nairobi or the Nairobi metropolitan region (Figure 3.1). The region has a total area of 30,390 Km^2 which is 5.5% of Kenya's total area with an estimated total population of more than 7 million people based on the 2019 population census. Most of the population is concentrated in Nairobi which has the highest concentration of infrastructure. The study area is reported to contribute up to 50% of Kenya's Gross Domestic Product (Kithakye, 2011).

The 35 urban centres where samples were collected included; Nairobi city centre, Westlands, Karen, Langata, Buruburu, Ruai, Eastleigh, Nairobi West, Imara Daima, Kasarani, Kawangware, Ruaka, Parklands, Kiambu town, Thika, Kenol, Ruiru, Kikuyu, Wangige, Limuru, Githunguri, Juja, Gatundu, Kajiado, Isinya, Matasia, Ngong, Ongata Rongai, Kiserian, Kitengela, Kangundo, Athi river, Syokimau, Machakos, Tala and Mlolongo. The study area has an altitude range of between 1,400m above sea level in the eastern region to 2,300m above sea level in the northern and western region. The main economic activities include manufacturing, mining, real estate development, agriculture, entrepreneurship and pastoralism. Majority of the products under study were processed and distributed within the study area.

Figure 3.1: Map of the Study Area showing sampling sites in the counties of Nairobi, Kiambu, Kajiado and Machakos (UN HABITAT, 2010)

3.2 Sample size

The sample size of the study was based on a formula by Cochran (1977)

$$n = \frac{Z\alpha^2 Pq}{L^2}$$

Where: Zα is 1.96, P, the prevalence of *Listeria monocytogenes* in milk and milk products and meat and meat products was taken as 5.6% and 14% based on studies conducted in Ethiopia by Seyoum *et al*. (2015) and Alsheikh *et al*. (2012) respectively, q is 1-P, L is precision at 0.025. This gave a sample size of 325 and 185 for milk and milk products and meat and meat products respectively. During sampling, a total of 570 samples were collected in the study area; 350 milk and milk products and 220 meat products.

3.3 Study design

The study design was cross sectional where milk and meat products namely, yoghurt, ice cream, short life pasteurized milk, long life pasteurized milk, milk from vending machines, milk powder, *mala*, ham, brawn, salami, polony and ready to eat meat bites were collected from retail markets in urban centres within Nairobi and surrounding counties between March 2017 and October 2018. The criterion for choosing the urban centres was based on availability of a retail market. In some cases, however, not all products were available in the selected centres.

After collection, the samples were properly labelled and placed in a cool box containing ice packs and transported to the research laboratory at the Department of Public Health, Pharmacology and Toxicology at the University of Nairobi for analysis. Aseptic techniques were observed to avoid contamination of the sample

from the collection site to the laboratory. Where analysis was not possible immediately, the samples were stored under refrigeration temperatures until required.

3.4 Sample preparation and analysis

Aseptic techniques were observed before opening the packaging material of the samples. This was mainly done by swabbing the bench and the packaging material with 70% alcohol and also by using gloves.

3.4.1 Enrichment and culturing

The enrichment and culturing was done as per the Food and Drug Administration (FDA)/ Bacteriological Analytical Manure (BAM) procedure (2011) with slight modifications. In this method, 25 g of the meat products and 25 ml of the milk products were placed in sterile stomacher bags after which 225 ml of Listeria enrichment broth (CM0862B Oxoid® UK) with supplements (SR0141E Oxoid® UK) prepared as per the manufacturer's instructions was added. Homogenization was done using a stomacher machine (Stomacher 400 Lab Blender) for 1 minute in normal speed after which the samples were incubated for 48 hours at 30°C (Sekonic pocketcorder incubator, Japan). A loopful of the enriched sample was then sub cultured for 24 hours at 37°C in Listeria selective agar (CM0856B Oxoid® UK) containing selective supplements (SR0140E Oxoid® UK). Grey colonies with a black surrounding were identified as possible *Listeria* colonies. Gram staining was conducted using the recommended protocol and gram-positive short rods were tentatively identified as belonging to the genus *Listeria*. Four distinct colonies from each plate were stored in cryotubes containing 10% skimmed milk (LP0031B Oxoid®

UK) at a temperature of -20°C until required for biochemical tests, CAMP test, molecular speciation, determination of virulence genes, sequencing and antibiotic sensitivity.

3.4.2 Reviving of stored colonies

The colonies stored in skimmed milk were thawed and revived by streaking a loopful on Tryptone Soy Agar (TSA) base (CM0131B Oxoid® UK) and incubating for 24 hours at 37°C. Distinct white colonies, indicative of *Listeria* species were used for the biochemical tests, CAMP test and DNA extraction for PCR.

3.4.3 Biochemical tests

The tests performed included the oxidase test, catalase test and sugar fermentation test. The sugars tested were; L-Rhamnose, D-Mannitol, D-Xylose and α-methyl mannoside. The oxidase test was performed by touching and spreading an isolated *Listeria* colony on an oxidase disc (Himedia® India) and the reaction observed within 10 seconds. Production of blue colour on the disc was indicative of a positive reaction while absence of colour was a negative reaction.

The catalase test was performed by picking an isolated *Listeria* colony and exposing it to a drop of 3% hydrogen peroxide solution on a sterile petri dish. Production of bubbles of oxygen indicated a positive test while lack of oxygen bubbles was a negative test.

The sugar fermentation test was done by dissolving the required amount of each sugar in distilled water. The solutions were then dispensed into bijou bottles and intermittent sterilization done for a period of 3 consecutive days for half an hour each

day. After the third day, the *Listeria* isolates were inoculated into the sugars and incubated at 37 °C for 48 hours after which methyl red was added for detection of pH change.

3.4.4 Christie, Atkins, Munch- Petersen (CAMP) test

This test was performed by streaking β hemolysin producing *Staphylococcus aureus* in blood agar along a vertical axis in the petri dish. This was followed by streaking the *Listeria* isolates at right angles, but not touching, the *S. aureus* and incubating the plates at 37 °C for 24 hours after which presence of enhanced hemolysis of the *Listeria* isolates in the vicinity of *S. aureus* was checked. *Streptococcus agalactiae* was used as a positive control.

3.4.5 Extraction of DNA from *Listeria* colonies

This was performed by placing the *Listeria* colonies in eppendorf tubes containing 200µl distilled water for DNA extraction and boiling at 100°C for 10 minutes in a water bath (Monday *et al*., 2007). The boiled suspension was then let to cool before being centrifuged (Eppendorf centrifuge 5424R, Hamburg, Germany) at 15,000 rpm for four minutes. The supernatant was transferred in a new DNAase/ RNAase free tube and stored at -20 °C until required for PCR analysis.

3.5 Genus determination by Polymerase Chain Reaction

A singleplex PCR was performed to determine the genus of the isolates. The *prs* gene, which is specific for strains of the genus *Listeria*, was targeted for amplification using primer sequences first described by Doumith *et al.* (2004). The primer forward and

reverse sequences, (F: 5′-GCTGAAGAGATTGCGAAAGAAG-3′ and R: 5′-CAAAGAAACCTTGGATTTGCGG-3′), were used to amplify a 370 bp region of the *prs* gene. The PCR amplification was carried out as per the conditions described by Mazza *et al.* (2015). Briefly, each PCR reaction mixture (25 µl) consisted of 2.5µl of 10X PCR buffer, a final concentration of 0.4µM for each primer, 1U of Taq DNA Polymerase (New England BioLabs® Inc.), 0.25µl of 100 mM $MgCl_2$, 2.0 µl of 2.5 mM of each dNTP and 5µl of DNA lysate template. All PCR reagents were supplied by New England BioLabs (NEB, USA) except for the primers which were supplied by Inqaba Biotec ™ (South Africa). The amplification was carried out in a PCR thermal cycler (Applied biosystems ™ Veriti 96 well thermal cycler) with an initial denaturation step at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 40 seconds, annealing at 58 °C for 30 seconds, followed by a final extension at 72 °C for 5 minutes. PCR products were electrophoresed on 1.5% (w/v) agarose gels in TAE (Tris–acetate–ethylenediamine tetra acetic acid) buffer, stained with ethidium bromide (0.05mg/µl) and visualized under ultraviolet (UV) light and the images acquired by the UVP Gelmax® imager.

3.6 Speciation by Multiplex Polymerase Chain Reaction

A multiplex PCR was done to identify the six species of Listeria (*L. monocytogenes, L. innocua, L. ivanovii, L. grayi, L.seeligeri and L.welshimeri*) among the isolates. The PCR protocol described by Mazza *et al*. (2015) for species identification was followed. The forward and reverse primer sequences, amplicon sizes and target genes are as indicated in Table 3.1.The reaction was performed in a final reaction volume of 25µl containing 2.5µl of 10X PCR buffer, 1U of Taq DNA Polymerase (New England

BioLabs® Inc.), 0.25µl of 100 mM MgCl2, 2.0 µl of 2.5mM of each dNTPs, 5µl of DNA lysate template and the primers for each species specific gene at the given concentration. The amplification was carried out in a PCR thermal cycler (Applied biosystems ™ Veriti 96 well thermal cycler) with an initial denaturation step at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 40 seconds, annealing at 58 °C for 30 seconds, followed by a final extension at 72 °C for 5 minutes. PCR products were electrophoresed on 1.5% (w/v) agarose gels in TAE (Tris–acetate–ethylenediamine tetra acetic acid) buffer, stained with ethidium bromide (0.05mg/µl) and visualized under ultraviolet (UV) light and the images acquired by the UVP Gelmax® imager.

Table 3.1: Target genes, primer pairs sequences and amplicon sizes for the six *Listeria* species (Mazza *et al.*, 2015)

Species	Target Gene	Primer pair sequences	Amplicon size (bp)
L. monocytogenes	*Lmo1030*	F:5′-GCTTGTATTCACTTGGATTTGTCTGG-3′ R: 5′-ACCATCCGCATATCTCAGCCAACT-3′	509
L. ivanovii	*namA*	F: 5′-CGAATTCCTTATTCACTTGAGC-3′ R: 5′-GGTGCTGCGAACTTAACTCA-3′	463
L. innocua	*Lin0464*	F: 5′-CGCATTTATCGCCAAAACTC-3′ R: 5′-TCGTGACATAGACGCGATTG-3′	749
L. grayi	*Oxidored -uctasi*	F:5′-GCGGATAAAGGTGTTCGGGTCAA-3′ R: 5′-ATTTGCTATCGTCCGAGGCTAGG-3′	201
L. seelingeri	*Lmo0333*	F: 5′-GTACCTGCTGGGAGTACATA-3′ R: 5′-CTGTCTCCATATCCGTACAG-3′	673
L. welshimeri	*scrA*	F: 5′-CGTGGCACAATAGCAATCTG-3′ R: 5′-GACATGCCTGCTGAACTAGA-3′	281

3.7 Gene sequencing and phylogenetic analysis of *Listeria monocytogenes*

DNA sequencing reactions of identified *Listeria monocytogenes* isolates was commercially done by Inqaba Biotec ™, South Africa. Both the forward and reverse

primers were used to ensure adequate coverage. DNA sequences were obtained in ABI format after which editing and creating of a consensus sequence from the forward and reverse sequences was done using BioEdit Sequence Alignment Editor Software® version 7.2.5. To compare consensus sequences of the isolated *L. monocytogenes* with reference isolates available in the GenBank database, sequence similarity was performed using the NCBI nucleotide BLAST search. Multiple sequence alignment and phylogenetic analysis of the DNA sequences was performed using the MEGA X Software. The phylogenetic analysis was carried out by subjecting the aligned sequences to the Maximum Likelihood (ML) method and reliability of the tree was estimated by the bootstrap method where a 1000 bootstrap value was used.

3.8 Serotyping of *Listeria monocytogenes* by Polymerase Chain Reaction

Confirmed *Listeria monocytogenes* isolates were serotyped by multiplex PCR using primers and conditions described by Doumith *et al.* (2004). All the primers used were acquired from Inqaba Biotec ™ (South Africa). The primer sequences, target genes, serotypes and amplicon sizes are as indicated in Table 3.2.

Table 3.2: Target genes, primer pair sequences and amplicon sizes for *Listeria monocytogenes* serotypes (Doumith *et al.*, 2004)

Serovar specificity	Target Gene	Primer pair sequences	Amplicon size (bp)
1/2a, 1/2c, 3a, and 3c	*Lmo0737*	F: 5′-AGGGCTTCAAGGACTTACCC-3′ R: 5′-ACGATTTCTGCTTGCCATTC-3′	691
1/2c and 3c	*Lmo1118*	F: 5′- AGGGGTCTTAAATCCTGGAA-3′ R: 5′-CGGCTTGTTCGGCATACTTA-3′	906
1/2b, 3b, 4b, 4d, and 4e	*ORF2819*	F: 5′-AGCAAAATGCCAAAACTCGT-3′ R: 5′- CATCACTAAAGCCTCCCATTG-3′	471
4b,4d, and 4e	*ORF2110*	F: 5-′AGTGGACAATTGATTGGTGAA-3′ R: 5′-CATCCATCCCTTACTTTGGAC-3′	597

The amplification was performed in a PCR thermal cycler (Applied biosystems ™ Veriti 96 well thermal cycler) with a final volume of 25µl containing 2.5µl of 10X PCR buffer, 1U of Taq DNA Polymerase (New England BioLabs® *Inc.*), 0.25µl of 100 mM $MgCl_2$, 2.0 µl of 2.5mM of each dNTPs, 5µl of DNA lysate template and each primer with a final concentration as described by Doumith *et al.* (2004). The amplification was performed with an initial denaturation step at 94 °C for 3 minutes, 35 cycles of 94 °C for 40 seconds, annealing temperature of 53 °C for 1 minute and 15 seconds, extension at 72 °C for 1 minute and 15 seconds and one final cycle of 72 °C for 7 minutes. PCR products were electrophoresed on 1.5% (w/v) agarose gels in TAE (Tris–acetate–ethylenediamine tetra acetic acid) buffer, stained with ethidium bromide (0.05mg/µl) and visualized under ultraviolet (UV) light and the images acquired by the UVP Gelmax®

3.9 Determination of *Listeria monocytogenes* virulence genes

The presence of the virulence genes *inlA, inlB, inlC, inlJ, hlyA, actA* and *iap* in the *Listeria monocytogenes* isolates was determined through multiplex PCR using primers and PCR cycling conditions as previously described by Liu *et al.* (2007) for the internalin genes and Osman *et al.* (2016) for the rest of the virulence genes. All the primers used were acquired from Inqaba Biotec ™ (South Africa). The target genes, sequences and amplicon sizes are as shown in Table 3.3.

Table 3.3: Target genes, primer pair sequences and amplicon sizes for *Listeria monocytogenes* virulence genes

Target Gene	Sequence	Amplicon size (bp)	Reference
inlA	F: 5′-ACGAGTAACGGGACAAATGC-3′ R: 5′-CCCGACAGTGGTGCTAGATT-3′	800	Liu *et al.*, 2007
inlB	F: 5′-TGGGAGAGTAACCCAACCAC-3′ R: 5′-GTTGACCTTCGATGGTTGCT-3′	884	Liu *et al.*, 2007
inlC	F: 5′-AATTCCCACAGGACACAACC-3′ R: 5′-CGGGAATGCAATTTTTCACTA-3′	517	Liu *et al.*, 2007
inlJ	F: 5′-TGTAACCCCGCTTACACAGTT-3′ R: 5′-AGCGGCTTGGCAGTCTAATA-3′	238	Liu *et al.*, 2007
hlyA	F: 5′-GCAGTTGCAAGCGCTTGGAGTGAA-3′ R: 5′-GCAACGTATCCTCCAGAGTGATCG-3′	456	Osman *et al.*, 2016
actA	F: 5′-CGCCGCGGAAATTAAAAAAAGA-3′ R: 5′-ACGAAGGAACCGGGCTGCTAG-3′	839	Osman *et al.*, 2016
iap	F: 5′-ACAAGCTGCACCTGTTGCAG-3′ R: 5′-TGACAGCGTGTGTAGTAGCA-3′	131	Osman *et al.*, 2016

All the amplifications were performed in a PCR thermal cycler (Applied biosystems ™ Veriti 96 well thermal cycler) with a final volume of 25µl containing 1U *Taq* DNA (New England Biolabs®), 2.5µl of 10X PCR buffer, 0.25µl of 100 mM $MgCl_2$, 2.0 µl of 2.5mM of each dNTPs, 5µl of DNA lysate template and 1.6µM of each *inlA*

primer, 1μM of each *inlB* primer, 1.2μM of each *inlC* primer and 0.8μM of each *inlJ* primer. DNA from *Listeria monocytogenes* strain ATCC 19115 serotype 4b and sterile distilled water without DNA were incorporated in the reactions as positive and negative controls, respectively. The amplification was performed with an initial denaturation temperature at 94 °C for 2 minutes, 30 cycles at 94 °C for 20 seconds, annealing at 55 °C for 20 seconds and extension at 72 °C for 50 seconds and 1 cycle at 72 °C for 2 minutes.

The virulence genes *hlyA*, *actA* and *iap* were determined as per the procedure and conditions described by Osman *et al.* (2016). The amplification was performed in a final volume of 25μl containing 5μl of template DNA, 0.5μM of each primer pair, 2.5μl of 10X PCR buffer, 0.25μl of 100 mM, $MgCl_2$, 2.0 μl of 2.5mM of each dNTPs, and 1U Taq DNA. The samples were subjected to an initial denaturation step at 95°C for 2 minutes followed by 35 cycles of 15 seconds at 95°C, annealing for 30 seconds at 60°C and extension at 72°C for 90 seconds and a final extension at 72 °C for 10 minutes. PCR products were electrophoresed on 1.5% (w/v) agarose gels in TAE (Tris–acetate–ethylenediamine tetra acetic acid) buffer, stained with ethidium bromide (0.05mg/μl) and visualized under ultraviolet (UV) light and the images acquired by the UVP Gelmax®

3.10 Antibiotic sensitivity testing

All *Listeria monocytogenes* isolates were tested for their susceptibility to antibiotics that are mainly used in veterinary and human therapy using the Kirby Bauer disc diffusion method. Three pure colonies of *Listeria monocytogenes* isolated from

Tryptone Soy Agar (TSA) (CM0131B Oxoid® UK) were suspended in physiological saline solution and the turbidity adjusted to 0.5 McFarland standard using a set of McFarland tubes. A sterile swab was dipped into the bacterial cell suspension and used to inoculate a Muller Hinton agar plate by swabbing over the entire surface to obtain a confluent growth. Antibiotic impregnated discs (Oxoid®) containing Erythromycin (E/15µg), chloramphenicol (C/30 µg), ciprofloxacin (CIP/30 µg), streptomycin (S/10µg), trimethoprim sulfamethoxazole (SXT/30 µg), penicillin (P/10), gentamycin (GEN/30 µg) and tetracycline (TE/30 µg) were applied on the surface of the inoculated plates and incubated at 37 °C for 24 hours. Zones of inhibition were measured and interpretation of susceptible, intermediate and resistant categories done on the basis of the Clinical and Laboratory Standards Institute Guidelines for gram positive bacteria (2018). *Listeria monocytogenes* ATCC19115 was used as the quality control strain.

3.11 Data analysis and presentation

Data from the isolation and speciation of *Listeria* species and *Listeria monocytogenes* was entered into the Statistical Package for Social Scientists (SPSS 20.0) for descriptive statistics on the frequency of *Listeria* species isolated from the various foods. The diameters of the zones of inhibition for the antimicrobial susceptibility tests were recorded in millimeters and susceptible, intermediate and resistant categories assigned. Chi square tests for association were done to test for the relationship between the isolated microorganism, food type and location. The p value cut off for significance was 0.05. Data on multiplex PCR and electrophoresis was presented in Figures which depict the regions of DNA separation based on the

Listeria species while descriptive data on the prevalence rates and percentages are presented in form of tables and graphs.

CHAPTER FOUR : RESULTS

4.1. Prevalence of *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs

4.1.1 Growth in Listeria Selective Agar

From the 570 milk and meat samples collected and analysed through bacteriology, 49 samples (8.59%) had isolates that showed growth characteristics similar to those of *Listeria spp* namely small grey colonies with a black surrounding in Listeria Selective Agar and small white colonies when purified in Tryptone Soy Yeast Agar as shown by the arrows in Figure 4.1 and 4.2. Twenty-one (42.86%) of the isolates were from milk and milk products while the rest (57.14%) were from meat products. On gram staining of these colonies, gram positive short rods indicative of *Listeria spp* were observed as indicated in Figure 4.3.

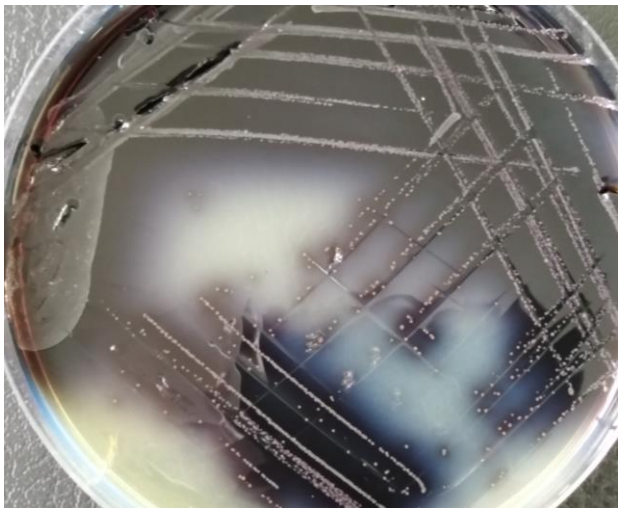

Figure 4.1: Small grey colonies and a black surrounding indicative of the growth of *Listeria spp* in Listeria Selective Agar (CM0856B Oxoid® UK) containing selective supplements (SR0140E Oxoid® UK)

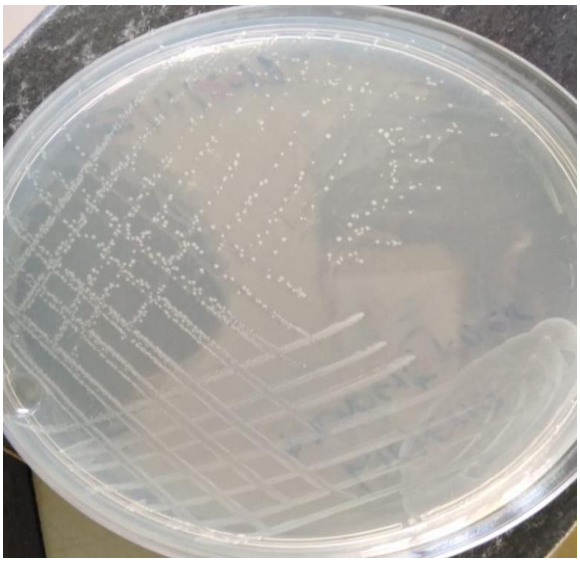

Figure 4.2: Small white colonies indicative of growth of Listeria species in Tryptone Soy Agar (CM0131B Oxoid® UK)

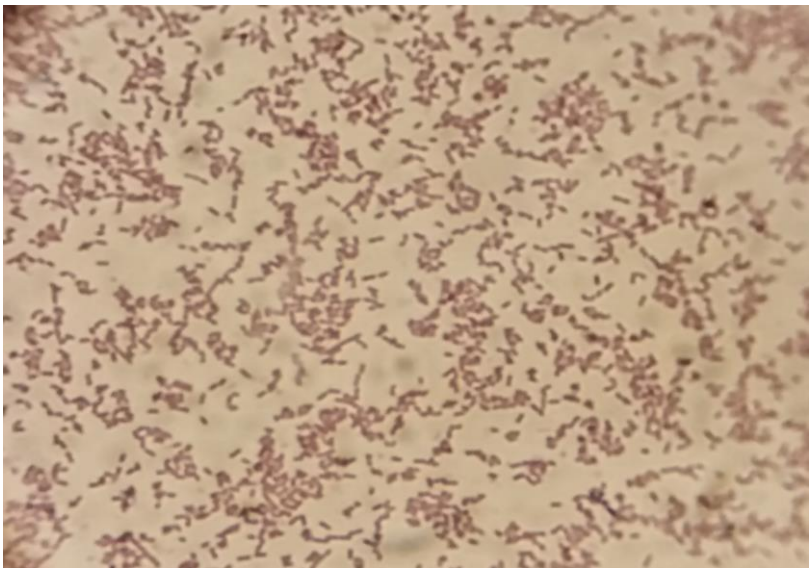

Figure 4.3: Gram stained *Listeria* species under x1000 magnification showing the gram-positive short rods

4.1.2 Biochemical tests

All the 49 *Listeria* isolates were positive for the Catalase test and negative for the Oxidase test indicative of *Listeria spp* (Figure 4.4). The results for the sugar fermentation tests were non-conclusive.

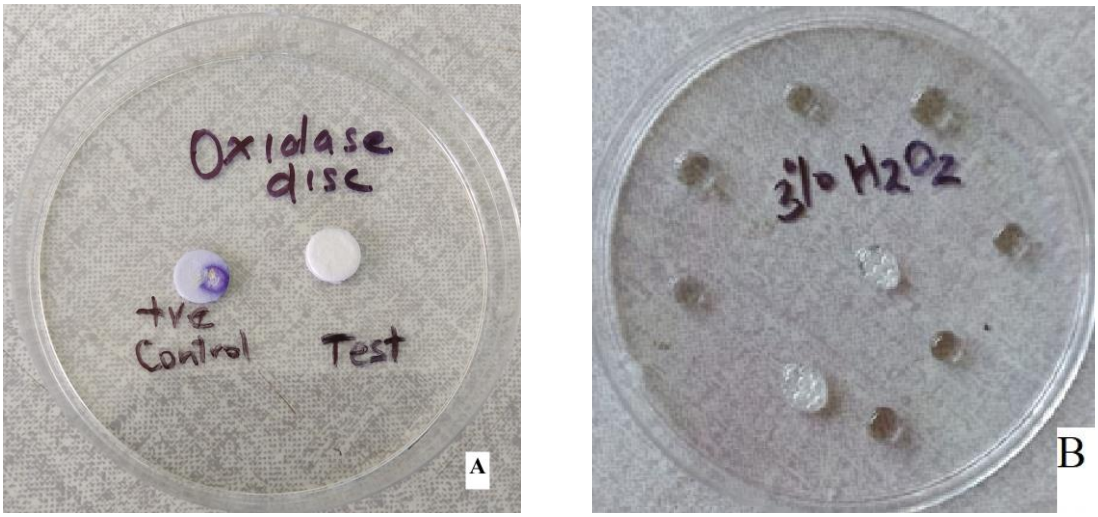

Figure 4.4: Negative Oxidase test (A) and positive Catalase test (B) for *Listeria spp*

4.1.3 Christie, Atkins, Munch- Petersen (CAMP) test

The results of the test revealed that 38.77 % (19/49) of the isolates had a zone of hemolysis in the vicinity of *Staphylococcus aureus* indicating that they were either *L. monocytogenes* or *L. seelingeri.* The rest of the isolates showed no hemolysis indicating they either belonged to other *Listeria* species or were non-pathogenic *Listeria monocytogenes*.

4.1.4 Confirmation of *Listeria* genus by Polymerase Chain Reaction

The DNA from the 49 *Listeria* isolates was analysed through PCR and all isolates were confirmed to belong to the genus *Listeria* after amplification of a 370 bp region of the *prs* gene (Figure 4.5). Of the twenty-one (42.85%) isolates obtained from milk and milk products, 4.76% was from milk powder (1/21), 4.76% from short life pasteurized milk (1/21), 14.29% from long life milk (3/21) and 76.19% from milk bought from dispensing/vending machines (16/21). The rest, 28/49 (57.14%) were obtained from meat and meat products namely; ham 2/28 (7.14%), brawn 13/28 (46.43%), polony 8/28 (28.57%), salami 1/28 (3.57%) and ready to eat meat bites 4/28 (14.28%). There were no isolates from *mala*, yoghurt, ice cream, cheese and milk cream. The overall prevalence of *Listeria* species in sampled milk and meat products is as shown in Table 4.1.

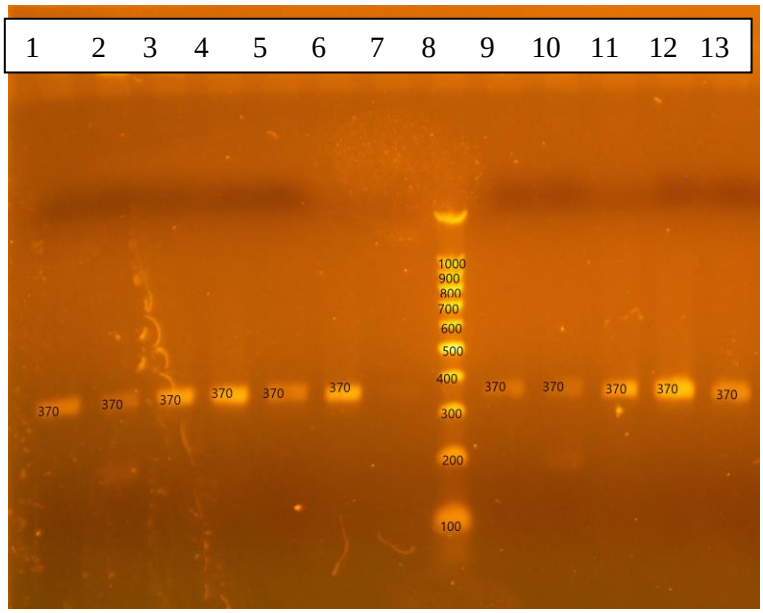

Figure 4.5: Ethidium bromide stained 1.5% agarose gel electrophoresis of Listeria isolates in singleplex PCR using prs gene (370 bp). Lane 1-5, 9-13 products amplified from DNA of *Listeria* isolates. Lane 6- positive control, Lane 7- Negative control, Lane 8- 100bp DNA ladder.

Table 4.1: Overall prevalence of *Listeria* species in meat and milk products

Product Type	No. of samples collected	No. of Listeria positive samples
Milk powder	17	1(5.8%)
Short life milk	67	1(1.5%)
Long life milk	65	3(4.6%)
Dispenser milk	36	16(44.4%)
Mala	27	0(0%)
Ice cream	24	0(0%)
Yoghurt	109	0(0%)
Cheese and milk cream	5	0(0%)
Polony	27	8(29.6%)
Brawn	73	13(17.8%)
Ham	37	2(5.4%)
Salami	6	1(16%)
Ready to eat meat bites	77	4(5.1%)
Total	**570**	**49(8.60%)**

Test for statistical association between the *Listeria* species and product type was done and it was determined that there was a significant association between the product type (meat and milk) and presence of *Listeria* species ($\chi^2 = 7.78$, df =1, $p<0.05$). Tests for strength of the association using the odds ratio showed that *Listeria* species was twice as likely to be isolated in meat products than in milk and milk products (OR= 2.28).

Stratification of the *Listeria spp* isolates per County of sample origin showed that Kajiado County had a relatively higher incidence based on the sample size (11.5%) as compared to Nairobi (8.1%), Kiambu (7.3%) and Machakos (10.5%) (Table 4.2). However, tests for association between the *Listeria spp* isolates and the County of

sample collection did not find any significant statistical association (χ^2 =1.68, df=3, p>0.05).

Table 4.2: *Listeria* species isolates per county

County	No. of samples collected	*Listeria* Species			Percent positive
		Negative	Positive		
			Meat	Milk	Total
Nairobi	221	203	16	2	18	8.1%
Kiambu	205	190	5	10	15	7.3%
Kajiado	87	77	6	4	10	11.5%
Machakos	57	51	1	5	6	10.5%
Total	**570**	**521**		**49**		

4.1.5 Confirmation of *Listeria* species by Polymerase Chain Reaction

Speciation of the *Listeria* isolates was done through multiplex PCR and of the 49 isolates, 22 (44.9%) were confirmed as *Listeria monocytogenes* through the amplification of a 509 bp region of the *Lmo1030* gene (Figure 4.6). Of these *Listeria monocytogenes* isolates, a majority (17/22; 77.27%) were from milk and milk products while 22.72% (5/22) were from ready to eat meat products. The highest prevalence, 68.18 % (15/22), was from dispensed milk followed by polony and brawn (9.09%) while the lowest, 4.54% (1/22) was from short life milk, long life milk and ham. Of the other 27(55.1%) *Listeria spp* isolates, two (7.4%) were identified as *Listeria welshimeri* by amplification of a 281 bp region of the *scrA* gene while three (11.1%) were identified as *Listeria innocua* by amplification of a 749 bp region of the *Lin0464* gene (Table 4.3 and Figure 4.6). The rest of the isolates, (22/27) (81.5%) were unidentified *Listeria*.

Table 4.3: Prevalence of *Listeria* species in milk and meat products

Product Type	**No. of samples collected**	***L. monocytogenes***	***L. innocua***	***L. welshimeri***	**Unidentified *Listeria spp***
Milk Powder	17	0(0%)	0(0%)	0(0%)	1(5.8%)
Short life milk	67	1(1.5%)	0(0%)	0(0%)	0(0%)
Long life milk	65	1(1.5%)	0(0%)	0(0%)	2(3.1%)
Dispenser milk	36	15(42%)	0(0%)	0(0%)	1(2.8%)
Mala	27	-	-	-	-
Ice cream	24	-	-	-	-
Yoghurt	109	-	-	-	-
Cheese and Milk cream	5	-	-	-	-
Polony	27	2(7.4%)	0(0%)	1(3.7%)	5(18.5%)
Brawn	73	2(2.7%)	3(4.1%)	0(0%)	8(10.9%)
Ham	37	1(2.7%)	0(0%)	1(2.7%)	0(0%)
Salami	6	0(0%)	0(0%)	0(0%)	1(16.6%)
Ready to eat meat bites	77	0(0%)	0(0%)	0(0%)	4(5.2%)
Total	**570(100%)**	**22 (3.86%)**	**3(0.53%)**	**2(0.35%)**	**22(3.86%)**

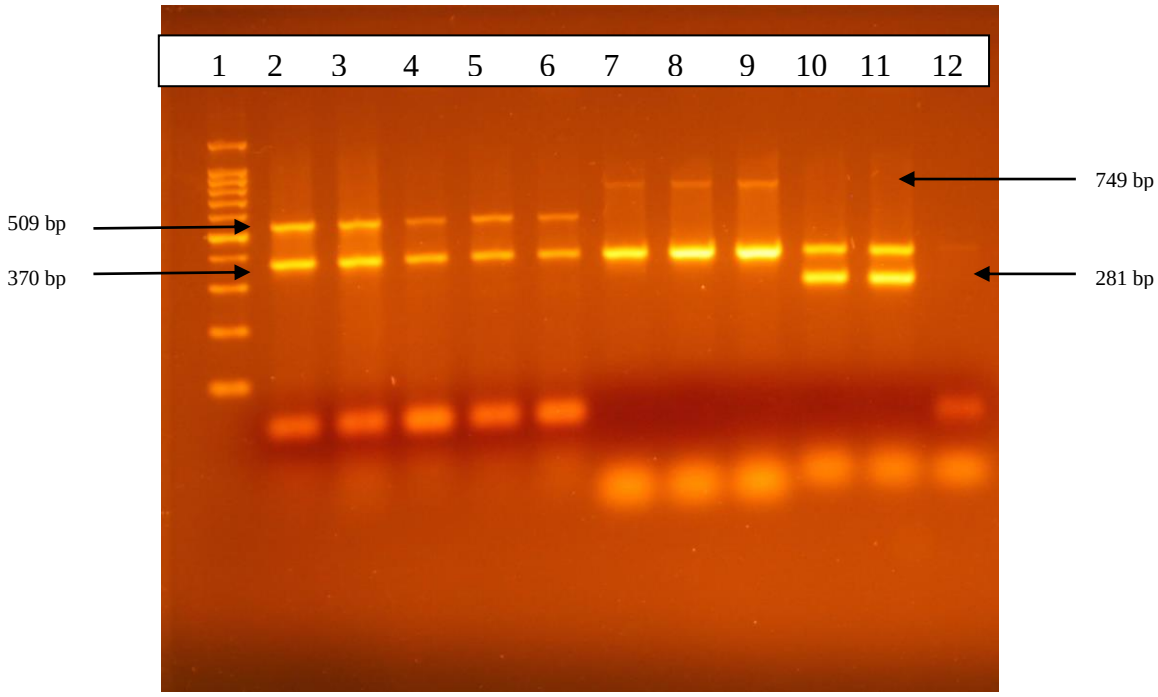

Figure 4.6: Ethidium bromide stained 1.5% agarose gel electrophoresis of Listeria species isolates in multiplex PCR using prs (370 bp), Lmo1030 (509 bp), scrA (281 bp) and Lin0464 (749 bp) genes. Lane 1, 100 bp DNA ladder, Lane 2 *Listeria monocytogenes* positive control (ATCC19115), Lanes 3, 4, 5, 6 products amplified from DNA of *Listeria monocytogenes* isolates, Lanes 7, 8, 9 products amplified from DNA of *Listeria innocua* isolates, Lane 10 and 11 products amplified from DNA of *Listeria welshimeri* isolates, Lane 12 negative control

Stratification of the *Listeria monocytogenes* isolates per County of origin was as follows; Nairobi (1.8%), Kiambu (3.9%), Kajiado (6.9%) and Machakos (7%) (Table 4.4). No statistical association was found between the *Listeria monocytogenes* isolates and the product type (χ^2 =2.43, df =1, p>0.05) and the *Listeria monocytogenes* isolates and the region of collection (χ^2 = 6.19, df =3, p>0.05).

Table 4.4: *Listeria monocytogenes* isolates per county

County of origin	**No. of samples collected**	***Listeria monocytogenes***				
		Negative	**Positive**			**Percent positive**
			Meat	**Milk**	**Total**	
Nairobi	221	217	2	2	4	1.8%
Kiambu	205	197	1	7	8	3.9%
Kajiado	87	81	2	4	6	6.9%
Machakos	57	53	0	4	4	7%
Total	**570**	**548**	**5**	**17**	**22**	**3.86%**

The overall prevalence of *Listeria monocytogenes* from the collected samples was 3.86% (22/570).

4.2 Major Serotypes of *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs

The results of the multiplex PCR confirmed that all the 22 isolates belonged to *Listeria* species through amplification of a 370 bp region of the *prs* gene (Figure 4.7). Twenty one of the twenty-two isolates (95.45%) carried both the *ORF2110* and *ORF2819* genes, characteristic of *L. monocytogenes* serotypes 4b, 4d or 4e. Of these isolates, 71.43% (15/21) were from milk sold through vending machines, 9.52% (2/21) from polony and an isolate each (4.76%) from packaged long-life milk, packaged short life milk, brawn and ham. The remaining isolate (1/22) (4.55%), carried the *ORF2819* gene only specific for serotypes 1/2b, 3b, or 7 and was isolated from brawn (Table 4.5). The other genes i.e. *Lmo0737* and *Lmo1118* were not detected (Figure 4.7; Table 4.5).

Table 4.5: Serotype distribution of isolated *Listeria monocytogenes*

Product Type	**No. of *L. monocytogenes* isolates (%)**	***L. monocytogenes* Serotypes**											
		1/2a	**1/2b**	**1/2c**	**3a**	**3b**	**3c**	**4a**	**4b**	**4c**	**4d**	**4e**	**7**
Short life milk	1(4.55)	-	-	-	-	-	-	-	+	-	+	+	-
Long life milk	1(4.55)	-	-	-	-	-	-	-	+	-	+	+	-
Dispenser milk	15(68.18)	-	-	-	-	-	-	-	+	-	+	+	-
Polony	2(9.1)	-	-	-	-	-	-	-	+	-	+	+	-
Brawn	2(9.1)	-	+	-	-	+	-	-	-	-	-	-	+
Ham	1(4.55)	-	-	-	-	-	-	-	+	-	+	+	-
TOTAL	**22(100%)**												

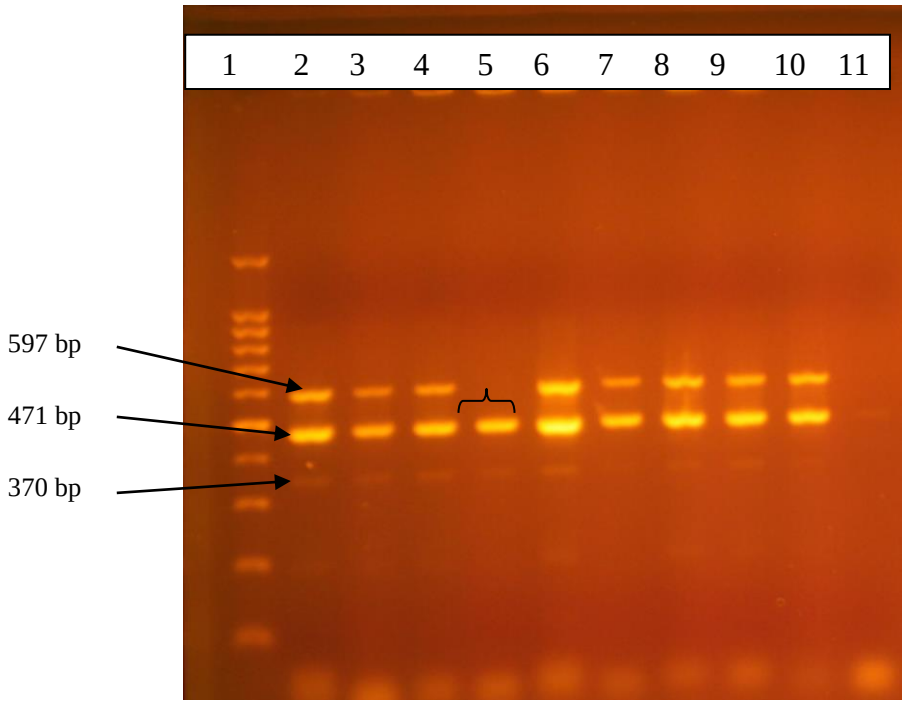

Figure 4.7: **Ethidium Bromide stained 1.5% Agarose gel electrophoresis of DNA fragments generated by multiplex PCR using the *prs* gene for serovar identification, (370 bp), and *ORF2819* (471 bp) and *ORF2110* (597 bp) genes for serotype identification.** Lane 1- 100bp DNA ladder, Lane 2- *Listeria monocytogenes* positive control (ATCC 19115), Lanes 3, 4, 6-10, *Listeria monocytogenes* isolates that had both the *ORF2819* and *ORF 2110* genes representing serotype 4b, 4d or 4e, Lane 5- *Listeria monocytogenes* with only the ORF 2819 gene representing serotype 1/2b, 3b or 7, Lane 11-Negative control. Fragments for two genes; *Lmo0737* and *Lmo1118* were not detected**.**

4.3 Virulence factors present in *Listeria monocytogenes* isolated from milk and meat products in Nairobi and its environs

The *Listeria monocytogenes* isolates were screened for the presence of seven virulence genes namely, *inlA, inlB, inlC, inlJ, hlyA, actA* and *iap* through multiplex PCR. The results showed that all the isolates possessed the *inlA, inlC, hlyA, iap* and *actA* genes. The *inlJ* gene was detected in 21/22 (95.45%) isolates. The gene was not detected from one isolate that was collected from dispensed milk. The *inlB* gene was detected in 10/22 (45.45%) isolates; nine from dispensed milk and one from brawn. The rest of the isolates (55.55%), majority of which were from milk collected from dispensers, did not possess the gene (Table 4.6; Figure 4.8; Figure 4.9).

Table 4.6: Virulence genes distribution in isolated *Listeria monocytogenes*

Product Type	No. of isolates	Virulence genes						
		inlA	***inlB***	***inlC***	***inlJ***	***hlyA***	***iap***	***actA***
Short life milk	1	+	-	+	+	+	+	+
Long life milk	1	+	-	+	+	+	+	+
Dispenser milk	15	+	+(9/15)	+	+(14/15)	+	+	+
Polony	2	+	-	+	+	+	+	+
Brawn	2	+	+(1/2)	+	+	+	+	+
Ham	1	+	-	+	+	+	+	+
TOTAL	**22**	**22**	**10**	**22**	**21**	**22**	**22**	**22**

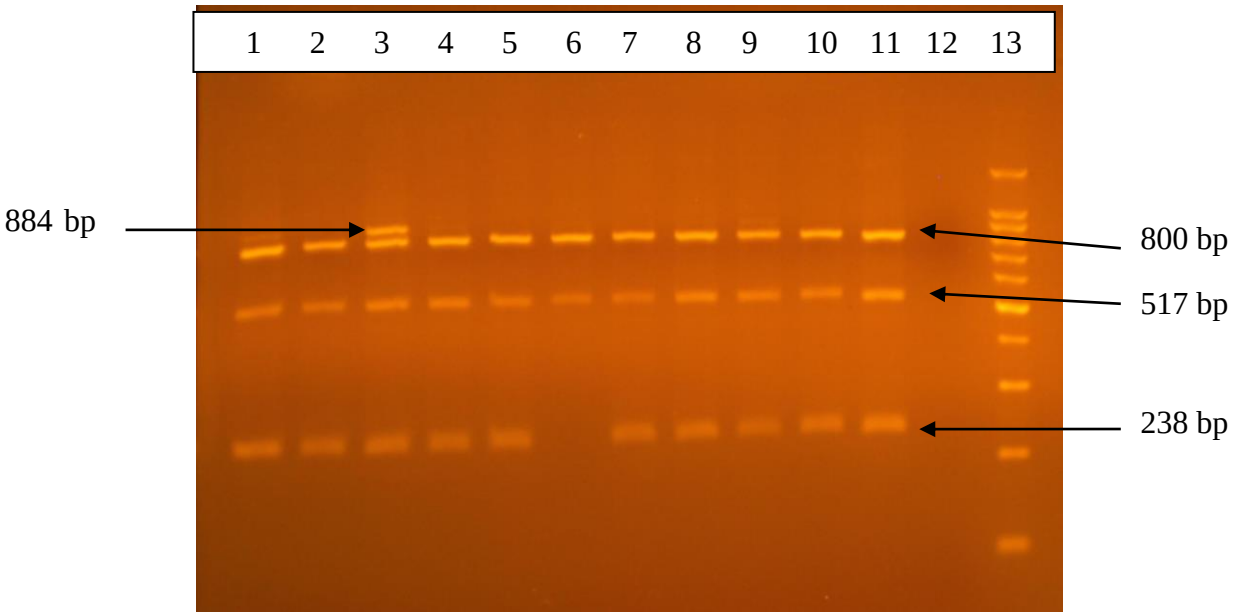

Figure 4.8: Ethidium bromide stained 1.5% Agarose gel electrophoresis of DNA fragments generated by multiplex PCR for the inlA (800 bp), inlB (884 bp), inlC (517 bp) and inlJ (238 bp) virulence genes. Lane 1 and Lane 3- *Listeria monocytogenes* isolates that had all the four genes, Lanes 2, 4-11, *Listeria monocytogenes* isolates that had *inl*A, *inl*C and *inl*J genes, Lane 6- one isolate that did not possess *inl*J, Lane 12- negative control, Lane 13- 100 bp ladder.

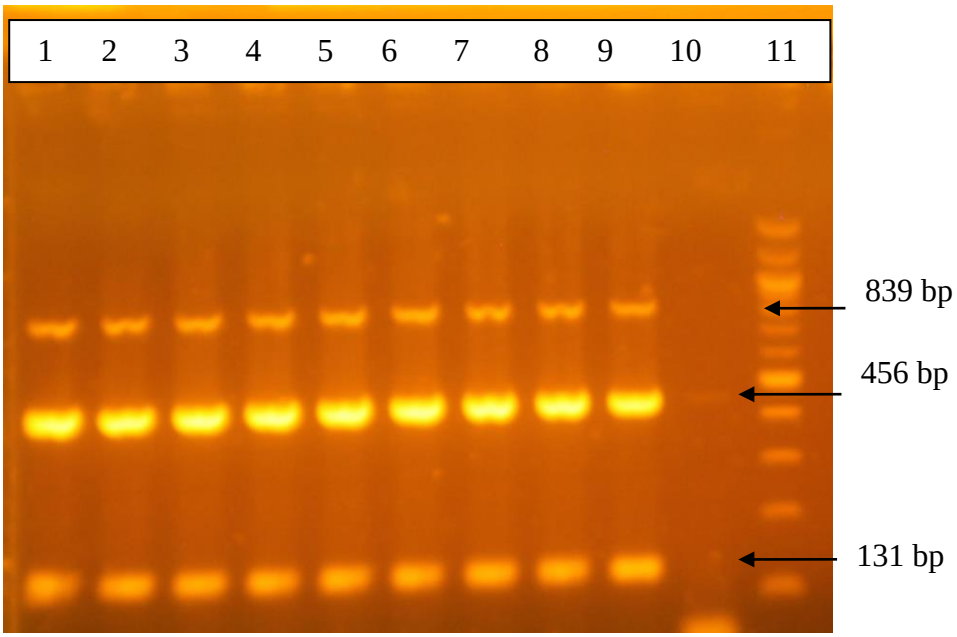

Figure 4.9: Ethidium bromide stained 1.5% Agarose gel electrophoresis of DNA fragments generated by multiplex PCR for the actA (839 bp), iap (131 bp) and hlyA (456 bp) virulence genes. Lane 1-8- *Listeria monocytogenes* isolates with the three virulence genes, Lane 9, *Listeria monocytogenes* positive control (ATCC 19115), Lane 10- negative control, Lane 11, 100 bp ladder.

4.4 The phylogenetic relationship of the isolated *Listeria monocytogenes* from milk and meat products in retail markets in Nairobi and its environs

4.4.1 *Lmo 1030* gene sequencing from *Listeria monocytogenes* isolates

Of the 22 *Listeria monocytogenes* isolates, only sequencing reactions for 15 isolates, targeting the *lmo1030 gene,* were successfully carried out as shown in Appendix I. The other seven isolates did not pass the quality assurance tests. Consensus sequences created from the forward and reverse DNA sequences of the 15 isolates using BioEdit Sequence Alignment Editor Software ® version 7.2.5 are as shown in Appendix II. Results for the sequence similarity that was carried out using the Basic Local Alignment Search Tool (BLASTn) showed that the isolates had a high identity of between 98%-99% with *L. monocytogenes* JI816, a serotype 4b strain isolated from a food processing plant, and *L. monocytogenes* R2-502, a serotype 1/2b strain isolated from food, in the USA (Table 4.7).

4.4.2 Phylogenetic analysis

Phylogenetic analysis carried out using MEGA X software after multiple sequence alignment revealed that the isolated *L. monocytogenes* was clustered into two main clades made up of a mixture of milk and meat isolates. A strong evolutionary relationship (Boot strap value>70%) was found between two isolates from milk (LMO154 and LMO155) collected from milk dispensers in Matasia and Kiserian respectively. The strong relationship was also found between two isolates from meat (LMM64 and LMM213) isolated from polony and brawn collected from retail

markets in Kiambu and Ngong respectively and processed by the same company (Figure 4.10).

The other organism clusters were supported by very low bootstrap values and therefore the output may not be very reliable (Figure 4.7).

Table 4.7: *Listeria monocytogenes* isolates, their sequenced homologues and percentage identity obtained from NCBI gene bank using BLASTn

Isolate ID	Source of the isolate	Homologue obtained by BLASTn	% identity of isolates with homologue
LMO28	Milk dispensing machine	*L. monocytogenes* JI816	507/509 (99%)
LMO32	Milk dispensing machine	*L. monocytogenes* JI816	498/508 (98%)
LMO113	Milk dispensing machine	*L. monocytogenes* JI816	483/484 (99%)
LMO154	Milk dispensing machine	*L. monocytogenes* JI816	501/508 (99%)
LMO155	Milk dispensing machine	*L. monocytogenes* JI816	501/508 (99%)
LMO193	Milk dispensing machine	*L. monocytogenes* JI816	506/508 (99%)
LMO199	Milk dispensing machine	*L. monocytogenes* JI816	506/509 (99%)
LMO229	Milk dispensing machine	*L. monocytogenes* JI816	505/506 (99%)
LMO338	Milk dispensing machine	*L. monocytogenes* JI816	508/509 (99%)
LMO339	Milk dispensing machine	*L. monocytogenes* JI816	508/509 (99%)
LMOM64	Polony	*L. monocytogenes* JI816	502/505 (99%)
LMOM77	Brawn	*L. monocytogenes* JI816	501/503 (99%)
LMOM83	Polony	*L. monocytogenes* JI816	505/509 (99%)
LMOM116	Ham	*L. monocytogenes* JI816	505/506 (99%)
LMOM213	Brawn	*L. monocytogenes* R2-502	507/509(99%)

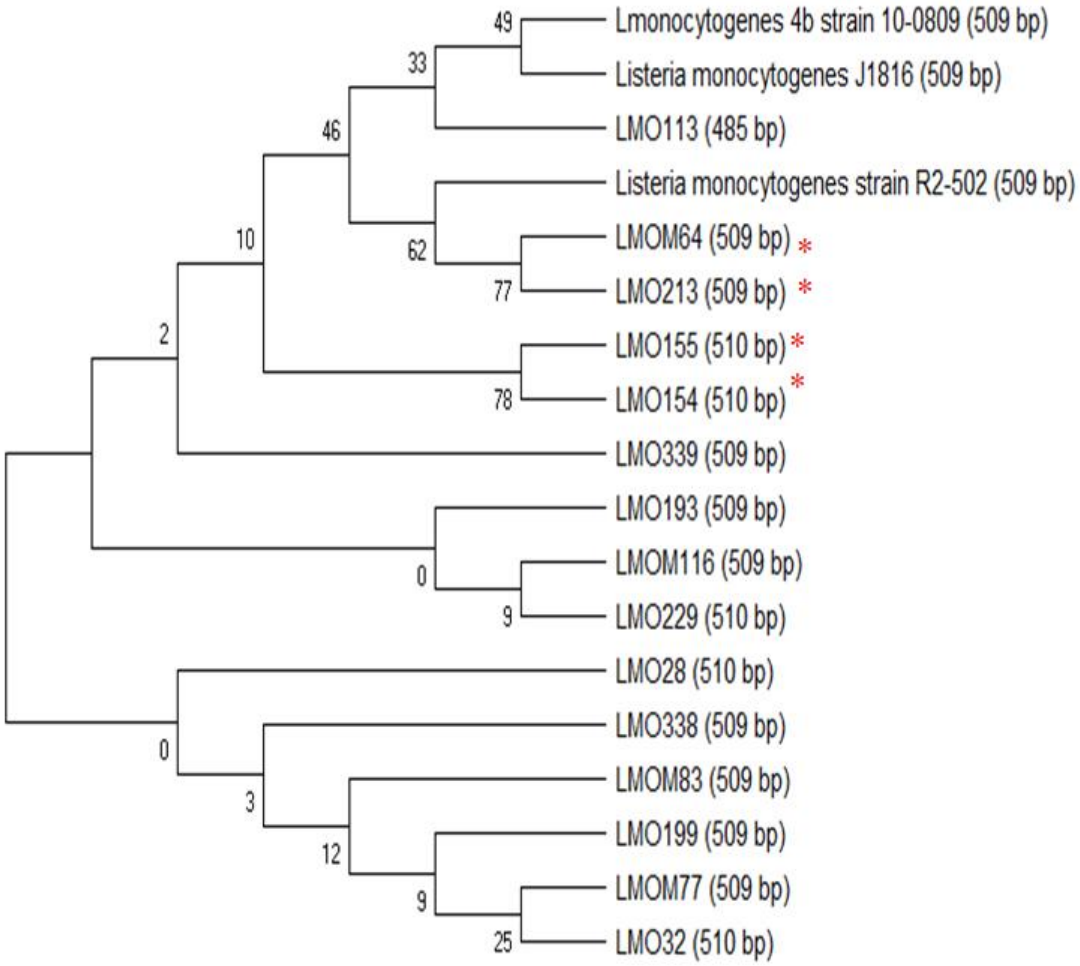

Figure 4.10: Molecular phylogenetic tree showing relationship between the 15 Listeria monocytogenes isolates from meat and milk products and 3 reference isolates from the gene bank. The relationship was inferred by using the Maximum Likelihood method based on the Tamura Nei model. All analysis was performed in MEGA X.

4.5 The antibiotic susceptibility patterns of *Listeria monocytogenes* isolated from milk and meat products in retail markets in Nairobi and its environs

The phenotypic antibiotic characteristics for the *Listeria monocytogenes* isolates was analysed using eight antibiotics namely penicillin G, tetracycline, chloramphenicol, gentamycin, streptomycin, ciprofloxacin, erythromycin, and trimethoprim sulfamethoxazole. The Kirby Bauer disk diffusion method was employed and the CLSI (2018) criteria for *Staphylococcus* species considered for analysis of resistant, intermediate and susceptible isolates since no criteria exists for *Listeria* susceptibility testing (Khen *et al*., 2014; Du *et al*., 2016).

All the 22 isolates, (100%) showed resistance to penicillin G, 9.09% (2/22) showed resistance to erythromycin and 4.54% (1/22) showed resistance to trimethoprim-sulfamethoxazole. One isolate from brawn (4.54%) had intermediate resistance to ciprofloxacin while 13 isolates (59%) had intermediate resistance to streptomycin.

On multidrug resistance, two isolates (9.09%) showed resistance to more than one antibiotic. One isolate from ham was resistant to three antibiotics (penicillin, erythromycin and trimethoprim-sulfamethoxazole) while the other from dispensed milk was resistant to penicillin and erythromycin. All isolates (100%) were susceptible to gentamicin, tetracycline, and chloramphenicol as indicated in Table 4.8.

Table 4.8: Antimicrobial susceptibility of *Listeria monocytogenes* from food to eight antimicrobials

	L. monocytogenes* isolates *(n=22)					
	Susceptible		**Intermediate**		**Resistant**	
Antimicrobial agent	**n**	**%**	**n**	**%**	**n**	**%**
Penicillin G (10 mg)	_	_	_	_	22	100
Gentamycin (30 mg)	22	100	_	_	_	_
Erythromycin (15 mg)	20	90.9	_	_	2	9.09
Tetracycline (30 mg)	22	100	_	_	_	_
Ciprofloxacin (30 mg)	21	95.45	1	4.54	_	_
Trimethoprime-sulfamethoxazole (30 mg)	21	95.45	_	_	1	4.54
Chloramphenicol (30 mg)	22	100	_	_	_	_
Streptomycin (10 mg)	9	40.9	13	59.1	_	_

CHAPTER FIVE : DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Prevalence of *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs

In the present study, *Listeria* species were detected in ready-to-eat milk and meat products with a prevalence of 8.59%. The prevalence was lower than what was reported in ready-to-eat foods of animal origin in Ethiopia which was between 25%-28.4 % (Derra *et al.*, 2013; Garedew *et al*., 2015; Seyoum *et al.*, 2015), in Turkey, 20.4%, (Sanlibaba *et al.,* 2018) and in Thailand, 16.5%, (Vongkamjan *et al.,* 2016). However, it was consistent with the 9.3% prevalence rate reported in Algiers, Algeria by Bouayad and Hamdi (2012).

The present study reports an overall prevalence of *Listeria monocytogenes* of 3.86% which is consistent with research carried out by Bouayad and Hamdi (2012) that reported a prevalence of 2.6% in Algiers, Algeria, Derra *et al.* (2013) reported a prevalence of 4.1% in Addis Ababa, Ethiopia, Morobe *et al*. (2009) reported a prevalence of 4.3% in Gaborone, Botswana and Gelbicova and Karpiskova (2009) reported a prevalence of 2.5% in the Czech Republic. Studies based on the ‘farm to fork concept’ have shown that contamination of the final product usually occurs during processing or at the retail level and less often at the farm or due to on farm factors (Hellström *et al*, 2010). Since the foods studied in the present study had already undergone significant heat processing, any microbial contamination could be attributed to post processing handling mostly during packaging or storage. Kurpas *et al*. (2018) reported that human factors played an important role in contamination of

the end product especially from employees who didn't comply with personal hygiene rules. This could have been the same scenario in the present study.

In the present study, the prevalence of *Listeria monocytogenes* in milk and milk products was higher than in ready-to-eat meat products with the highest prevalence being recorded in pasteurized milk from vending machines. The organism was also isolated from long life and short life pasteurized milk. Milk is one of the most important foods consumed by humans in addition to being a good culture medium for microorganisms including *Listeria monocytogenes* (Lee *et al.,* 2019). The process of pasteurization is meant to eliminate pathogens in milk and therefore the presence of *Listeria monocytogenes* in pasteurized milk may be due to contamination after pasteurization or faults in technology during pasteurization either due to inadequate temperature or a decreased pasteurization time (Navratilova *et al*, 2004; Lee *et al.,* 2019). These finding are consistent with findings by Sreeja *et al*. (2016) and Sheela and Shrinithivihahshini (2017) in India and Navratilova *et al*. (2004) in Czech Republic who reported presence of *Listeria monocytogenes* in pasteurized packaged milk. They are also consistent with what has been previously reported in Kenya on the inadequacy of pasteurization in packaged milk (Mwangi *et al*., 2000; Nato *et al.,* 2016).

It was noted during data collection that milk sold through vending machines was brought in using metallic cans from processors and dispensed to consumers. Contamination could have occurred through improper handling of this milk during transfer to the vending machines, inadequate cleaning of the vending machines and

the subsequent formation of structured communities of *Listeria monocytogenes* cells known as biofilms that are able to embed themselves on food processing surfaces and niches. These biofilms are able to resist biocides and stress conditions including cleaning and disinfection (Colagiorgi *et al*., 2017).

There was no isolation of *L. monocytogenes* from yoghurt, *mala* and ice cream. This finding is consistent with studies by Abrahão *et al*. (2008), Akman *et al*. (2004) and Mugampoza *et al*. (2011) who reported no isolation from ice cream and fermented milk products in Brazil, Turkey and Uganda respectively. The absence of *L. monocytogenes* in these products could have been due to use of Ultra High Temperature (UHT) processed milk in their production or low pH and effect of bacteriocins produced by lactic acid bacteria in the fermented products.

In the current study, the overall prevalence of *Listeria spp* was higher in meat products than in milk products. Apart from the species that were identified, there was also a significant portion of unidentified *Listeria* species which were mainly present in meat brawn, polony and ready to eat meat bites. These other *Listeria* could belong to any of the 12 species that were not tested for since the study was focusing on the six most common species of *Listeria* which are related with animal hosts. The other 12 *Listeria* species are mainly found in the environment; water, soil and decaying plant matter (Orsi and Wiedmann, 2016). Their presence in food therefore could indicate contamination of processing equipment.

5.1.2 Major serotypes of *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs

The study concluded that a majority of the *Listeria monocytogenes* isolates carried both the ORF 2110 and ORF 2819 genes characteristic of serovar 4b, 4d or 4e while one isolate carried the ORF 2819 gene only characteristic of serovar 1/2b, 3b, or 7. However, according to Doumith *et al*, (2004), serotypes 4d, 4e, 3b and 7 are infrequently isolated in foods and rarely reported as implicated in human listeriosis and therefore the most likely serotypes to have been isolated from the foods were 1/2b and 4b. These results are of great significance since among the 13 serotypes of *Listeria monocytogenes*, serotypes 1/2a, 1/2b and 4b account for 95% of listeriosis outbreaks worldwide (Kathariou, 2002; Montero *et al.*, 2015).

The most frequently isolated serotype from foods and the environment is serotype 1/2a accounting for more than 50% of the *L. monocytogenes* isolates while major outbreaks of human listeriosis have been caused by serotype 4b strains (Gilbreth *et al*, 2005). In a study by Goulet *et al* (2006) on 603 cases of human listeriosis in France between the years 2001 and 2003, it was reported that serotype 4b and 1/2b accounted for 49% and 20% of the cases respectively and were most likely the cause of central nervous system infections, maternal neonatal disease and bacteraemia. Liu (2013) indicated that serovar 4b strains have been shown to cause endemic human disease while serotypes 1/2a, 1/2b and 1/2c are responsible for sporadic listeriosis in humans.

Genotyping of the *L. monocytogenes* isolates is considered more specific than serotyping which lacks the desired specificity due to extensive antigen sharing among

the serotypes. Through genotyping, *Listeria monocytogenes* can be separated into four phylogenetic lineages (I-IV) that vary in their ecological, evolutionary and phenotypic characteristics including virulence (Orsi *et al*., 2011; Liu, 2013). From the present study, the isolated serotypes can be grouped into lineage I which is composed of serotypes 1/2b, 3b, 4b, 4d and 4e. This lineage has strains that are frequently associated with human clinical cases and predominantly linked to outbreaks involving invasive disease (Lomonaco *et al.*, 2015).

The results of the present study are consistent with studies conducted elsewhere in the world but with varying distribution in the three serotypes linked to human listeriosis. In a study conducted by Braga *et al.* (2017) on the serotype distribution in various foods collected in Montevideo, Uruguay, serotypes 1/2b and 4b were the most frequently identified with 1/2b having a slightly higher frequency of occurrence than 4b. In Chile, a study conducted by Montero *et al*. (2015) in a wide variety of ready to eat foods determined that serotype 4b was the most prevalent followed by 1/2a and 1/2b. In Iran, a study done by Ranjbar and Halaji (2018) determined that serotypes 4b, 1/2a and 1/2b were the most commonly isolated in foods while in Colombia, a descriptive and retrospective study conducted over a ten year period and in more than 1500 bacterial isolates from foods by Munoz (2012), established that serotype 4b was the most prevalent followed by serotype 1/2b. In Brazil, a study carried out over a twenty-year period by Vallim *et al.* (2015) in foods also established the predominance of serotype 4b.

In contrast, several studies have reported predominance of serotype 1/2a as the main serovar isolated in foods especially in the Northern hemisphere. A study conducted in the United States of America on 502 *Listeria monocytogenes* isolates by Gilbreth *et al.* (2005) reported majority of the isolates to be either serotype 1/2a or 1/2b. In Belgium, a study conducted by Coillie *et al.* (2004) showed that most of the isolates belonged to serotype 1/2a while in Tokyo, Japan, Shimojima *et al.* (2016) reported a low prevalence of *L. monocytogenes* (1.7%) with the most common serovar being 1/2a followed by 1/2b, 4b and 1/2c. In Wales, Meldrum *et al.* (2010) reported that 1/2a was the predominant serotype in ready-to-eat foods sampled from point of sale. Latorre *et al.* (2007) and Gelbicorva and Karpiskova (2009) reported similar findings in Italy and the Czech Republic respectively. The findings of this study are indicative of a threat faced by consumers of products where the two most common serotypes implicated in Listeriosis were isolated.

5.1.3 Virulence factors present in isolated *Listeria monocytogenes* from milk and meat products in retail markets in Nairobi and its environs

Listeria monocytogenes strains vary in their virulence potential with some being naturally virulent and having the ability to inflict high morbidity and mortality while others are non-virulent and are unable to infect the mammalian host (Soni *et al.*, 2014). As indicated in the results, the study was able to detect all the seven virulence genes tested for in the isolates though in varying proportions. The expression of major virulence factors that mediate the intracellular life cycle of *L. monocytogenes* is controlled by PrfA which activates transcription in response to environmental signals, including temperature and nutrient availability (Phelps *et al*., 2018). Some of these

virulence factors include Internalin A, a listerial surface protein that is required for the penetration of *L. monocytogenes* into epithelial cells (Doyle, 2001). It is also used as a species-specific marker for *Listeria monocytogenes* (Liu, 2007). Internalin B is also a listerial surface protein that also mediates the entry of *L. monocytogenes* into host cells. However, while internalin A targets the adhesion molecule E- cadherin, Internalin B targets the hepatocyte growth factor receptor Met. Met is expressed by a wide range of cells and this allows internalin B to mediate entry of bacteria in a large number of cell types and has been implicated in liver colonization after intravenous infection of mice while E-cadherin is only expressed by a limited number of cells of epithelial origin (Bonazzi *et al*, 2009; Pentecost *et al.*, 2010). It has also been reported that internalin B works synergistically with internalin A to promote fetoplacental infection (Pentecost *et al.*, 2010; Schuppler and Loessner, 2010)

All isolates from the present study had the *inl*C gene which encodes internalin C, a virulence marker that contributes to the post intestinal stages of *Listeria monocytogenes* infection. Internalin C is only present in pathogenic Listeria strains and is expressed in the cytoplasm, especially in the late stages of infection when bacteria are in the process of active intracellular spread (Vazquez-Boland *et al*., 2001). The gene that encodes for internalin J was detected in 95% of the isolates in the study. In a report by Sabet *et al.* (2005), internalin J was found to be directly involved in the passage of *Listeria monocytogenes* through the intestinal barrier and in the subsequent stages of infection and virulence (Liu, 2007). This could be due to the fact that it has been found to be produced and localized at the surface of bacteria present in the liver and blood of infected animals (Sabet *et al.*, 2008). It is present in

strains that are capable of causing human listerial outbreaks and mouse mortality but absent in avirulent, non-pathogenic strains (Liu, 2006).

The gene *hly*A encoding Listeriolysin O (LLO), a bacterial pore forming toxin was also detected in all the isolates in the present study. It is essential in the lysing of the vacuolar membrane and allowing *L monocytogenes* to escape into the cytoplasm of the cell (Doyle, 2001; Schuppler and Loessner 2010). Strains that don't carry the gene may be non-virulent or may have undergone spontaneous mutations (Usman *et al.*, 2016). Once the vacuolar membrane is lysed, LLO is recognized by enzymes in the cell cytoplasm which destroy it before it can damage the cell membrane (Doyle, 2001).

The actA protein induces the polymerization of actin in the infected cell cytoplasm to form polarized actin filaments which aid in the movement of bacterial cells within the cell and between cells (Liu, 2006). The protein is encoded by the *act*A gene which was present in all the isolates in the present study. The invasion associated protein (iap) was also detected in all the isolates from the study. This protein possesses murein hydrolase activity that is necessary for a late step in cell division (Doyle, 2001). The presence of all the virulence genes tested for in almost all the isolates indicates that the isolated *L. monocytogenes* is pathogenic and capable of causing clinical disease especially in susceptible individuals where the infective dose may range from 0.1-10 million colony forming units per gram (Farber *et al.*, 1996).

5.1.4 The phylogenetic relationship of the isolated *Listeria monocytogenes* from milk and meat products in retail markets in Nairobi and its environs

The results of the present study showed that DNA sequences from the 15 *Listeria monocytogenes* isolates had a high identity (98%-99%) with homologous sequences in the gene bank. These homologous sequences belonged to serotypes 4b and 1/2b, a confirmation of the serotyping results. These results are in agreement with a study done by Mohamed *et al* (2016) in Giza, Egypt on the prevalence and phylogenetic characterization of *L. monocytogenes* from processed meat where 6 isolates were found to have a high homology (98%-99%) with isolates in the gene bank.

A strong phylogenetic relationship was established between two isolates from milk (LMO154 and LMO155) and two isolates from meat (LMOM64 and LMOM213) (Bootstrap values >70). The isolates from milk were collected from milk vending machines in Matasia and Kiserian, towns that are approximately 6 kilometres apart and therefore it could be possible that they originated from the same milk supplier. This assertion is supported by a study conducted by Pantoja *et al*, (2012) in the USA on contamination of bulked milk, where it was reported that six of seven isolates from bulked milk were identical and from the same molecular type. The two isolates from meat were from brawn and polony, two entirely different products with distinct processing techniques. They were also collected from two different locations; Kiambu and Ngong. However, they were processed in the same factory and therefore the detection of *L. monocytogenes* in these products could be an indicator of cross contamination between product lines. According to a report by Kurpas *et al* (2018), *L.*

monocytogenes may form biofilms on the surfaces of processing equipment which may act as reservoirs for contamination.

5.1.5 The antibiotic susceptibility patterns of *Listeria monocytogenes* isolated from milk and meat products in retail markets in Nairobi and its environs

The results of the present study showed that all the 22 *Listeria monocytogenes* isolates were resistant to penicillin, a first line antibiotic against listeriosis. Penicillins are β lactam-based antibiotics that act by inhibiting bacterial cell wall synthesis. Resistance to penicillin in *Listeria monocytogenes* has been shown to occur through the acquisition of *penA* encoding gene, a known penicillin binding protein (PBP) that was first identified in *Neisseria meningitidis* (Srinivasan *et al*., 2005). The results are significant since currently penicillin or ampicillin combined with gentamicin form the reference therapy for listeriosis in humans (Aras and Ardiç, 2015).

The results are consistent with a study conducted by Kuan *et al*. (2017) on the antimicrobial resistance of *L. monocytogenes* isolated from vegetables in Malaysia where all isolates were found to be resistant to penicillin G. Similar findings were reported by Sanlibaba *et al.* (2018) in ready-to-eat foods in Turkey where all isolated strains were resistant to penicillin while Harakeh *et al*. (2009) reported that 90% of *L. monocytogenes* isolates from dairy products were resistant to penicillin in a Northeast region of Lebanon. In Ethiopia, Garedew *et al.* (2015) reported a slightly lower resistance (66.7%) while Issa *et al.*, (2011) reported that most *L. monocytogenes*

strains isolated from foods were resistant to penicillin and ampicillin. These results imply that treatment of active listeriosis cases using penicillin may not be effective and second line antibiotics may need to be considered. The results also confirm what has been published on the detection of antimicrobial residues and antimicrobial resistant organism in milk in Kenya (Orwa *et al.*, 2017; Kosgey *et al.*, 2018)

In other studies, however, *L. monocytogenes* has been reported to be susceptible to penicillin. In a study conducted by Wu *et al.* (2015) in China, penicillin G was found to be the only antibiotic to which all the 248 *L. monocytogenes* isolates were susceptible to. Similar findings were reported by Caplan *et al.* (2014) in food and clinical isolates of *L. monocytogenes* in Romania.

Two isolates from the present study were resistant to erythromycin, an antibiotic belonging to the macrolide group. These antibiotics act by inhibiting protein synthesis through binding to the 50s ribosomal subunit of bacteria (Luque-Sastre *et al*., 2018). The most common resistance mechanism to macrolides is due to the presence of rRNA methylases which are encoded by the *erm* genes. These enzymes methylate an adenine base and thus prevent the binding of the drug to the 50s ribosomal sub unit (Luque-Sastre *et al*., 2018). Among the resistance genes recognised in gram positive bacteria, only *erm*(A), *erm*(B) and *erm*(C) have been reported in Listeria species (Granier *et al.,* 2011). These genes have also been associated with mobile genetic elements mainly plasmid pAMβ1 and pIP501 originating from *Enterococcus faecalis* and *Streptococcus agalactiae* respectively (Wilson *et al.,* 2018). Resistance to macrolides and especially erythromycin can also be mediated through efflux pumps

such as *msr*(A) found in *Staphylococcus species* and *mef*(A) found in *Streptococcus pneumonia* (Morvan *et al.*, 2010; Granier *et al.*, 2011).

One isolate from the study was resistant to sulfamethoxazole trimethoprim, antibiotics that are used in combination due to their synergistic effect. They act on bacteria by inhibiting folic acid synthesis. Folic acid is essential for the synthesis of adenine and thymine, bases that are involved as structural components of nucleic acids (Luque-Sastre *et al*., 2018). Resistance to trimethoprim by *L. monocytogenes* has been attributed to the *dfr*D resistance encoding gene (Eliopoulos and Huovinen, 2001). This gene can be transferred by conjugative mobilization between *L. monocytogenes, Staphylococcus species* and *Escherichia coli* (Wilson *et al.*, 2018). The resistance of the isolates to erythromycin and sulfamethoxazole trimethoprim is an important finding since these drugs are second choice antibiotic used in the treatment of listeriosis especially in pregnant women or in people allergic to the first-choice drugs (Hof, 2004).

The development of resistance to erythromycin concurs with findings of Wu *et al.* (2015) in China where 3/248 isolates (1.2%) were found to be resistant to erythromycin. In Colombia, a study conducted by Ruiz-Bolivar *et al.* (2011) on 108 *L. monocytogenes* food isolates reported one isolate (0.9%) to be resistant to erythromycin while Caplan *et al.* (2014) reported intermediate resistance of *L. monocytogenes* isolated from food and infected humans to the same drug. In contrast, a study done by Sanlibaba *et al.* (2018) had all *L. monocytogenes* isolates susceptible

to erythromycin. Morobe *et al.* (2009) and Bryne *et al.* (2016) also reported similar findings in Botswana and Brazil respectively in isolates from food and vegetables.

The development of resistance to sulfamethoxazole trimethoprim concurs with findings of a study by Caplan *et al.* (2014) in food and human clinical isolates where one isolate was found to be resistant. In a study conducted in Turkey by Yucel *et al.* (2005), 66% of the *L. monocytogenes* isolates were found to be resistant to sulfamethoxazole and trimethoprim. Similar findings were reported by Conter *et al.* (2009) where out of 120 *L. monocytogenes* isolates, 14 showed resistance to at least one antibiotic including sulfamethoxazole trimethoprim. Contrary to the findings of the present study, Sakaridis *et al.* (2011), in a study conducted on the prevalence and antimicrobial resistance of *Listeria monocytogenes* in chicken slaughter houses in Northern Greece reported that all 55 isolates obtained were susceptible to various antimicrobials among them sulfamethoxazole trimethoprim. Similar findings were reported by Abdollahzadeh *et al*. (2016) in sea food and clinical isolates in Iran, and by Bryne *et al.* (2016) in isolates recovered from vegetables in Brazil and by Garedew *et al.* (2015) in ready-to-eat foods of animal origin in Ethiopia.

The results of the study also showed that 4.5% and 59% of the isolates had intermediate susceptibility to ciprofloxacin and streptomycin respectively. Ciprofloxacin is a fluoroquinolone, a group of antibiotics that act by inhibiting bacterial DNA replication. On the other hand, streptomycin, an aminoglycoside acts by inhibiting protein synthesis by binding to the 30s ribosomal subunit. Resistance to ciprofloxacin is mainly attributed to gene mutations within the quinolone resistance

determining regions of genes encoding the bacterial topoisomerase enzyme while resistance to streptomycin is associated with resistance gene *aad*6 (Liu *et al.*, 2007; Morvan *et al.*, 2010).

The results of the present study are consistent with findings by Wu *et al.* (2015) where 4.8% and 4% of the *L. monocytogenes* isolates from various foods were found to be resistant to streptomycin and ciprofloxacin respectively. Osaili *et al.* (2012) in a study on the occurrence of *L. monocytogenes* in brined white cheese in Jordan reported that the isolates were sensitive or of intermediate susceptibility to ciprofloxacin. Haubert *et al* (2016) in a study carried out in Southern Brazil on food and environmental samples reported that all isolates were susceptible to ciprofloxacin and 10% susceptible to streptomycin. Bryne *et al.* (2016) reported that their isolates were susceptible to both ciprofloxacin and streptomycin.

The isolates from the present study were all sensitive to gentamicin, tetracycline, and chloramphenicol. The sensitivity of the isolates to gentamicin is a significant finding since as indicated earlier, gentamicin is a first-choice drug against listeriosis in combination with penicillin. Similar findings have been reported by Caplan *et al.* (2014) in Romania, Sakaridis *et al.* (2011) in Northern Greece, Garedew *et al.* (2015) in Ethiopia and in Nigeria by Peter *et al.* (2016) where *L. monocytogenes* isolates from raw beef, pork and chicken sold in Makurdi metropolis were found to be susceptible to gentamicin and chloramphenicol. In Iran, Jamali *et al.* (2015) reported that all isolates from raw fish and open-air fish markets were susceptible to gentamicin, cefotaxime, kanamycin and pefloxacin. On the other hand, Prazak *et al.*

(2002) evaluated the antimicrobial susceptibility of 21 *L. monocytogenes* isolates from vegetables, packing sheds and water and was able to detect one isolate that was resistant to gentamycin. In India, Jeyasanta and Patterson (2016) indicated that 75.67% of isolates from sea foods were resistant to gentamycin while in Egypt, Mohamed *et al.* (2018) also reported resistance to gentamycin alongside chloramphenicol, erythromycin, amoxicillin and norfloxacin.

Several publications on the susceptibility of *Listeria monocytogenes* isolates to tetracycline have been written. In Turkey, a study done by Terzi *et al.* (2015) on the antibiotic susceptibility of *L. monocytogenes* from ready to eat foods reported that all isolates were susceptible to tetracycline. Similar findings were reported by Abdollahzadeh *et al.* (2016) in Iran and Caplan *et al.* (2014) in Romania. In contrast, Jamali *et al.* (2015) reported that 27.95% of *Listeria monocytogenes* isolates showed resistance to tetracycline. The same was recorded by Kevenk and Gulel (2016) in Turkey, Geradew *et al*. (2015) in Ethiopia, Peter *et al.* (2016) in Nigeria and Bryne *et al.* (2016) in Brazil. Tetracycline resistance is the most frequently reported resistance in *Listeria* species (Luque-Sastre *et al.,* 2018). It has been mainly attributed to mobile genetic elements carrying the tetracycline resistance genes *tet*(A), *tet*(K) and *tet*(L) which confer proton antiporter efflux pump proteins. It has also been associated with *tet*(M) and *tet*(S) genes which confer ribosomal protection proteins (Charpentier and Courvalin, 1999; Granier *et al.*, 2011).

As indicated earlier, all the isolates in the present study were susceptible to chloramphenicol, an antibiotic that inhibits protein synthesis in bacteria. The findings

are in agreement with publications by Caplan *et al.* (2014), Sanlibaba *et al.* (2015) and Abdollazadeh *et al.* (2016). Reports of resistance of *L. monocytogenes* to chloramphenicol have been published by Wu *et al.* (2015) in China, Garedew *et al.* (2015) in Ethiopia, Kevenk and Gulel (2016) in Turkey and Mohamed *et al.* (2018) in Egypt. Resistance to chloramphenicol is mainly attributed to inactivation of the drug by type A chloramphenicol acetyltransferases and the export of chloramphenicol by specific efflux proteins (Luque-Sastre *et al*., 2018). Multidrug resistance is when a single bacterium is resistant to more than one antibiotic. In the current study, two isolates (9.09%) were resistant to more than one antibiotic. This finding is of significance to human and animal health since the drugs the isolates were resistant to are used in the day to day treatment of bacterial infections and thus resistance may increase the mortality rate, the recovery time in hospitals and medical costs (WHO, 2018).

The first multidrug resistant strain of *L. monocytogenes* was isolated in France in 1988 from a patient suffering from meningoencephalitis. This strain was resistant to chloramphenicol, erythromycin, streptomycin and tetracycline (Poyart-Salmeron *et al*., 1990). Since then, many reports of multidrug resistant *L. monocytogenes* isolates from foods, clinical cases and the environment have been published. In a study done in Turkey by Sanlibaba *et al.* (2018), it was reported that all the 17 *L. monocytogenes* isolates from ready to eat foods were resistant to at least three antibiotics. In another study by Jamali *et al.* (2015) in the Northern region of Iran, 60% of the *L. monocytogenes* isolates from raw fish and fish market environments were found to be resistant to two or more antibiotics. Similarly, Wu *et al*. (2015) in China reported that

2.8% of isolates from raw retail foods were resistant to more than 10 antibiotics. Other studies by Prazak *et al.* (2002), Yucel *et al.* (2005), Yan *et al.* (2010), Sakaridis *et al.* (2011), Garedew *et al.* (2015) and Kevenk and Gulel (2016) have also reported the presence of multidrug resistant strains of *L. monocytogenes.*

Apart from the presence of resistance genes and mobile genetic elements namely; self-transferable plasmids, mobilizable plasmids and conjugative transposons, resistance to antibiotics by *L. monocytogenes* may also be attributed to other factors such as the extensive and indiscriminate use of antibiotics in humans and animals (Wilson *et al.*, 2018). In animals, antibiotics are used to prevent, control and treat illnesses as well as to enhance their growth (Lungu *et al.*, 2011). The constant encounter of *L. monocytogenes* to low levels of antibiotics and other antimicrobials in the food production chain may serve as a form of pre-exposure adaptation which subsequently allows the organism to resist higher levels of antibiotics (Olaimat *et al*., 2018).

Resistance may also be attributed to some foodborne organisms being intrinsically resistant to certain antibiotics due to their physiology. Organisms in the genus *Listeria* are naturally resistant to broad spectrum cephalosporins such as cefotaxime (Luque-Sastre *et al.*, 2018). In a publication on the antibiotic resistance of *Listeria monocytogenes* in food production by Lungu *et al* (2011), it was noted that the stressful environments that foodborne microorganisms encounter in food processing industries may also contribute to the development of resistance to antibiotics. Such stressful environments included the effect of physical stressors such as heat, chemical

stressors such as oxidants and biological stressors such as microbial antagonism where one microorganism is used to inhibit the growth of another for instance the use of lactic acid bacteria to control the growth of *L. monocytogenes* in foods (Kostrzynska and Bachand, 2006 ;Wesche *et al.,* 2009). In a study conducted by Al-Nabulsi *et al*. (2015) on the effects of osmotic pressure, acid or cold stresses on antibiotic susceptibility, it was established that exposure of *L. monocytogenes* to pH, cold and salt stresses increased their resistance to different antibiotics. A study by Faezi-Ghasemi and Kazemi (2015) also reported that the exposure of the exponential phase of *L. monocytogenes* to a concentration of 600 ppm of hydrogen peroxide and non-lethal heat (45°C) increased the organism's resistance to antibiotics. Other mechanisms that could contribute to resistance include; decreased outer membrane permeability, activation of efflux pumps and mutation in a ribosomal protein gene (Srinivasan *et al*., 2005).

5.2 Conclusions

The present study concludes that:

i). *Listeria monocytogenes* was present in milk and meat products sold in retail outlets in Nairobi and its environs with an overall prevalence of 3.86% (22/570). The incidence in milk and milk products was 77.27% while in meat products was 22.72%. The organism was isolated from pasteurized milk sold through vending machines, packaged pasteurized milk, polony, ham and brawn. Other *listeria* species identified were *Listeria welshimeri* (7.4%) and *Listeria innocua* (11.1%).

ii). *Listeria monocytogenes* in milk and meat products in retail outlets in Nairobi and its environs is present in serotypes 1/2b, 3b, 4b, 4d, 4e, or 7. Some of these serotypes have been reported to account for up to 95% of listeriosis cases worldwide.

iii). *Listeria monocytogenes* in milk and meat products in retail outlets in Nairobi and its environs is the virulent type and has a variety of virulence genes namely; *inlA*, *inlB*, *inlC*, *inlJ*, *hlyA actA* and *iap*, which aid in entry and spread within host cells in the body.

iv). There was no close genetic relationship between isolates except in two isolates from milk and two from meat.

v). *Listeria monocytogenes* in milk and meat products in retail markets in Nairobi and its environs is resistant to penicillin, erythromycin and trimethoprim sulfamethoxazole, drugs that are essential in the treatment of listeriosis. The isolates also had intermediate susceptibility to ciprofloxacin and streptomycin and susceptible to gentamicin, chloramphenicol and tetracycline.

5.3 Recommendations

i). Strict regulation of the processing and storage conditions of ready to eat foods of animal origin by health officials to ensure they reach consumers pathogen free.

ii). Proper regulation of the sale of milk through vending machines as they may be a source of a listeriosis outbreak.

iii). Observation of strict hygiene measures including the proper cleaning and disinfection of milk vending machines to avoid dispensing of contaminated milk.

iv). Proper regulation of the dispensing and administration of antibiotics in both humans and animals.

5.4 Areas for further studies

i) Identification of the 22 unidentified *Listeria* species from this study.

ii) Further analysis using more advanced serotyping techniques to identify the exact serotype for each of the isolates.

iii) Identification of the antibiotic resistance genes that may be present in the *Listeria monocytogenes* isolates from the study.

iv) Studies to be conducted to determine the prevalence of clinical listeriosis in both animals and man. There is currently scanty information on the same.

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APPENDICES

APPENDIX I: Approval of Research Proposal Letter

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 810901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School

DATE: 7th November, 2016

TO: Mr. Kevin K. Kabui
C/o Department of Zoological Sciences
Kenyatta University

REF: I84/29617/14

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

We acknowledge the receipt of your revised Research Proposal entitled **"Epidemiological Characterization and Antibiotic Sensitivity of Listeria monocytogenes Isolated from Foods of Animal Origin in Nairobi and its Environs, Kenya"** as per recommendations raised by the Graduate School Board of 7th October, 2016.

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation

As you embark on your data collection, please note that you will be required to submit to Graduate School completed supervision Tracking Forms per semester. The form has been developed to replace the progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

By copy of this letter, the registrar (Academic) is hereby requested to grant you Substantive registration for your Ph.D. studies.

Thank you.

REUBEN MURIUKI
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Department of Zoological Sciences
Registrar (Academic) Att; Mr. Likam

Supervisors

1. Prof. Michael Gicheru
   C/o Department of Zoological Sciences
   Kenyatta University
2. Prof. Samuel Arima
   Dept. of Public Health, Pharmacology & Toxicology
   C/o Department of Zoological Sciences
   University of Nairobi

RM/cao

APPENDIX II: Research License

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **730198**

Date of Issue: **06/November/2019**

RESEARCH LICENSE

This is to Certify that Dr.. KEVIN KABUI of Kenyatta University, has been licensed to conduct research in Kajiado, Kiambu, Machakos, Nairobi on the topic: Epidemiological Characterization and Antibiotic Sensitivity of Listeria monocytogenes isolated from foods of animal origin in Nairobi and its environs, Kenya. for the period ending : 06/November/2020.

License No: **NACOSTI/P/19/2604**

730198

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

APPENDIX III: Abstract of paper presented in the First International Food Safety Conference held in Kenyatta University from 20th -24th May 2019

ISOLATION OF LISTERIA SPECIES IN MILK AND MEAT PRODUCTS IN NAIROBI AND ITS ENVIRONS AND THE IMPLICATION IN FOOD SAFETY

Kabui, K. K [a] , Gathura, P. B [b], Nduhiu, J. G [b], Mainga, A. O [b] and **Gicheru, M. M [a]**

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[b] Department of Public Health, Pharmacology and Toxicology, P.O. Box 29053 Nairobi 00625, Kenya

***Corresponding author:** kinyua.kabui@ku.ac.ke

ABSTRACT

Food borne infections are an important public health concern worldwide with most being caused by pathogens that are zoonotic in nature. The main mode of transmission of these diseases is through consumption of contaminated food and water. Among the most common food borne infections is listeriosis which is caused by *Listeria monocytogenes*, a bacterium that is widely distributed in nature and which has been isolated in a wide array of foods. The disease is said to be the leading cause of death in reported cases of food poisoning with mortality rate of between 30% - 50%. It mainly affects pregnant women, neonates, the elderly and the immunocompromised. Most of the reported cases have been in developed countries, with little or no data in most developing countries, including Kenya. It is against this backdrop that a study was conducted in Nairobi and the surrounding Counties of Kiambu, Machakos and

Kajiado to determine the occurrence of Listeria species, especially *Listeria monocytogenes* in milk and meat products.

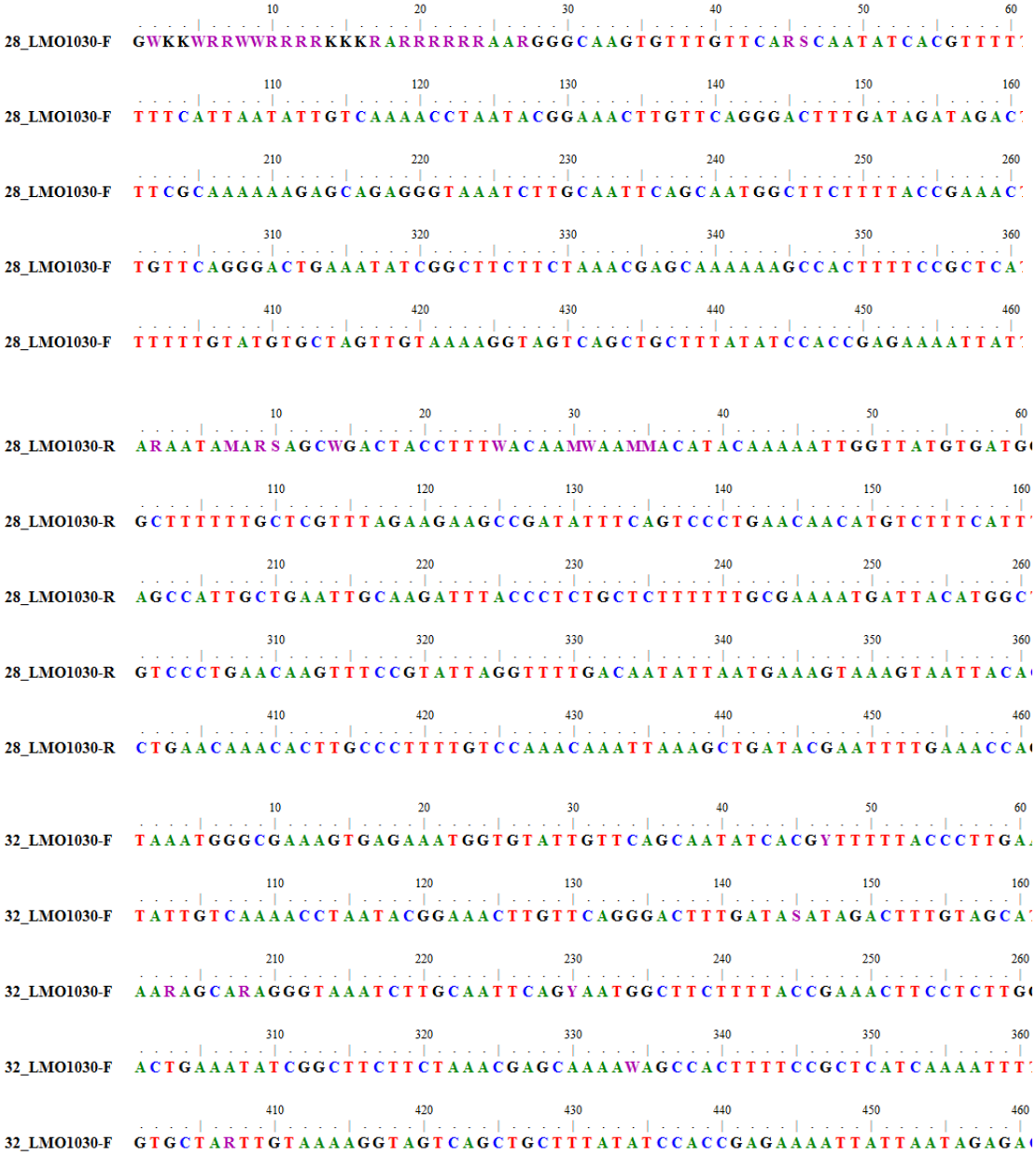
A total of 570 processed samples; 350 milk products and 220 meat products were collected from randomly selected retail markets in the study area and analyzed using conventional culture methods and PCR. Out of the 570 samples, 49 (8.59%) tested positive for Listeria species after amplification of a 370 bp region of the *prs* gene. Twenty one (42.8%) of these isolates were obtained from milk products namely; milk powder 1/17 (5.8%), short life pasteurized milk 1/66 (1.5%), long life pasteurized milk 3/62 (4.83%) and pasteurized milk from dispensing machines 16/20 (80%). The rest, 28/49 (57.2%) were obtained from meat products namely; ham 2/37 (5.4%), brawn 13/73 (17.8%), polony 8/27 (29.6%), salami 1/6 (16.7%) and ready to eat meat bites 4/77 (5.19%). There were no isolates from *mala*, yoghurt, ice cream, cheese and milk cream.

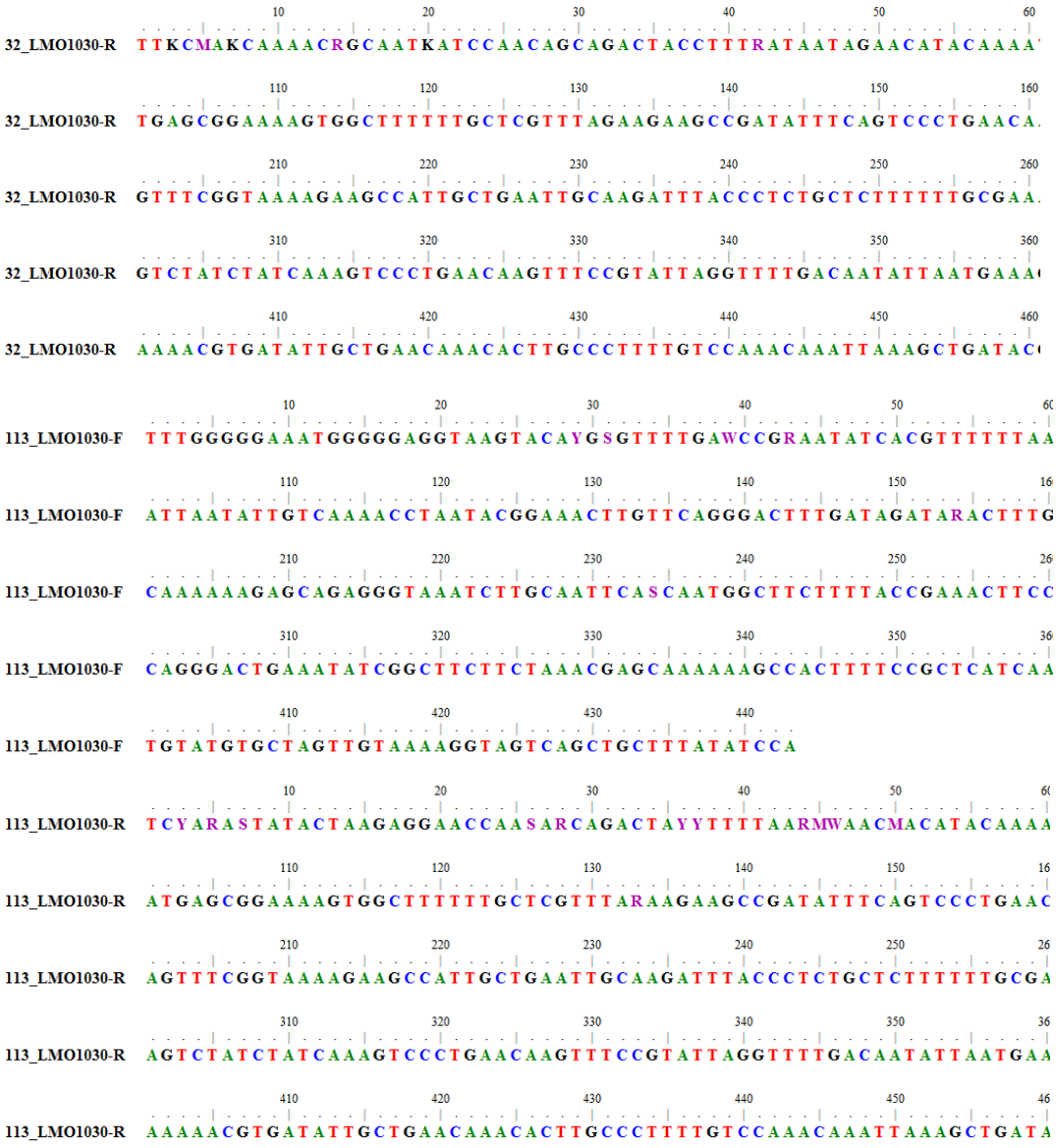
Speciation of the Listeria isolates was done through multiplex PCR and of the 49 isolates, 22 were confirmed as *Listeria monocytogenes* through the amplification of a 509 bp region of the *Lmo1030* gene. Of these *Listeria monocytogenes* isolates, 77.27% (17/22) were from milk products while 22.72% (5/22) were from meat products. The highest prevalence, 68.18 % (15/22) was from dispensed milk followed by polony and brawn (9.09%) while the lowest, 4.54% (1/22) was from short life milk, long life milk and ham. Of the other 27 Listeria genus isolates, two were identified as *Listeria welshimeri* by amplification of a 281 bp region of the *scrA* gene while three were identified as *Listeria innocua* by amplification of a 749 bp region of the *Lin0464* gene. The rest of the isolates, (22/27) were unidentified Listeria.

The overall prevalence of *Listeria monocytogenes* from the collected samples was 3.86% (22/570). These results have far reaching implications in terms of food safety to stakeholders in the food processing industry, human and animal health practitioners and the consumers of these products.

Keywords: Listeria, Ready to eat foods, *Listeria monocytogenes*

APPENDIX IV: DNA sequences of isolated *Listeria monocytogenes*

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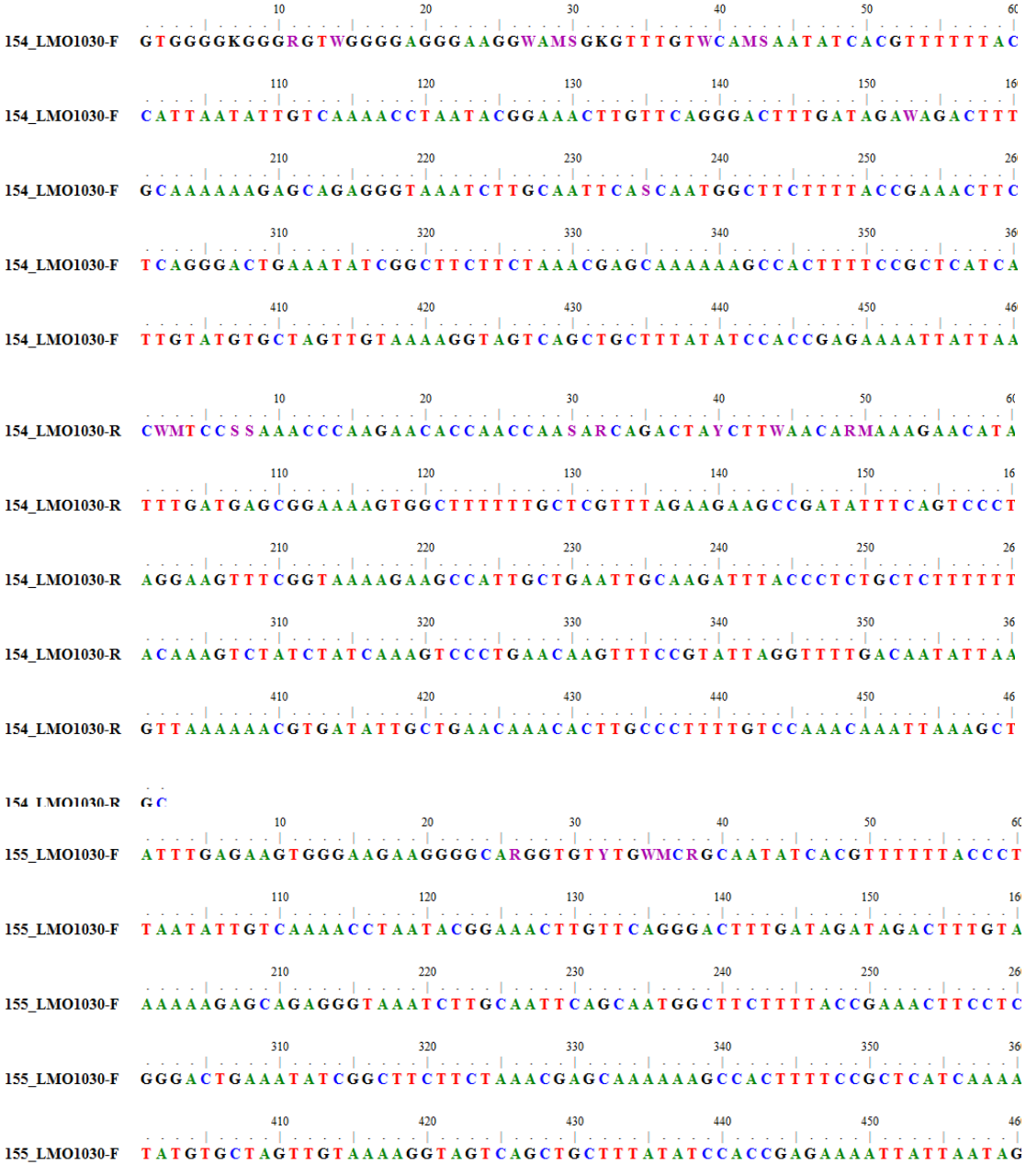
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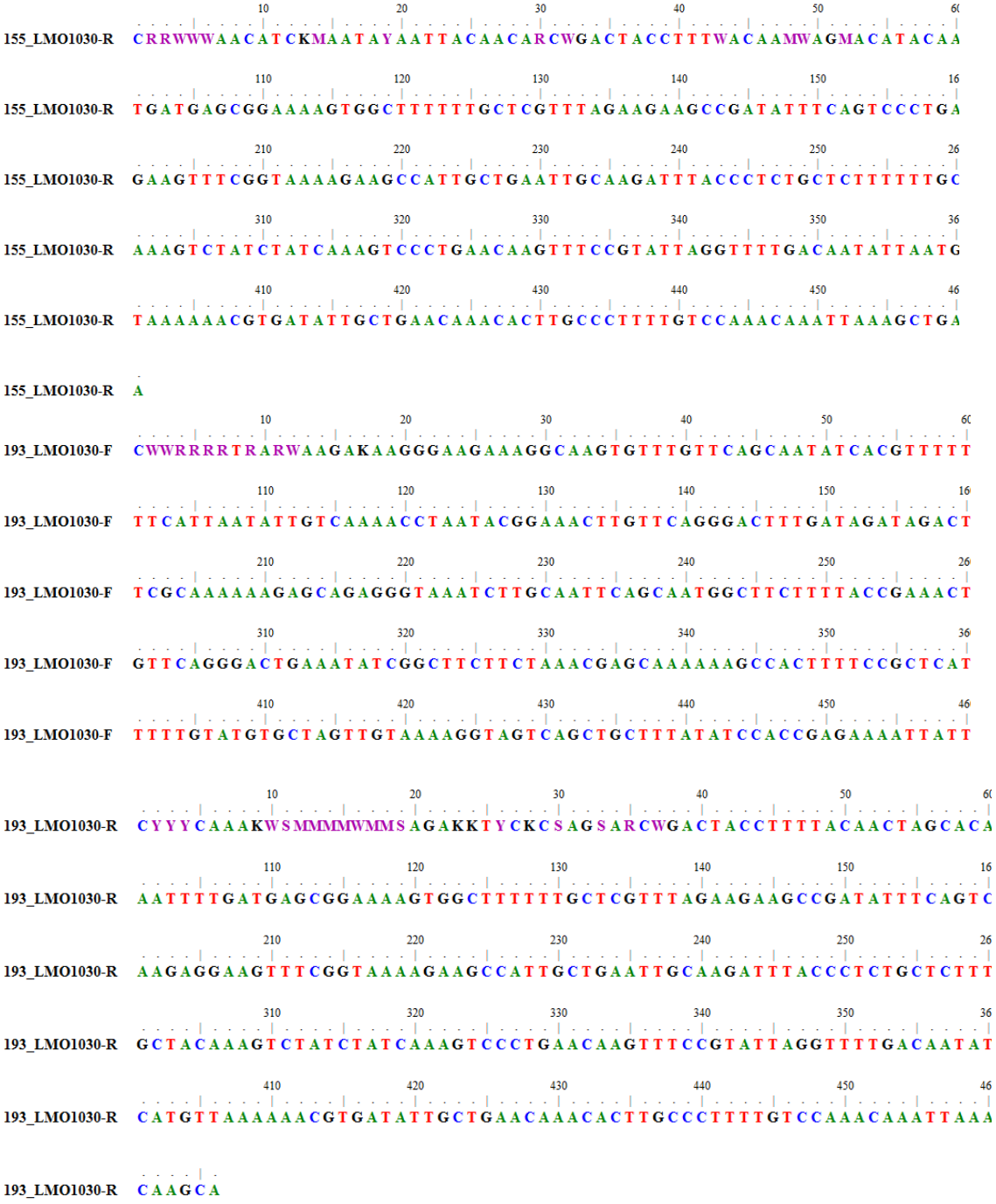
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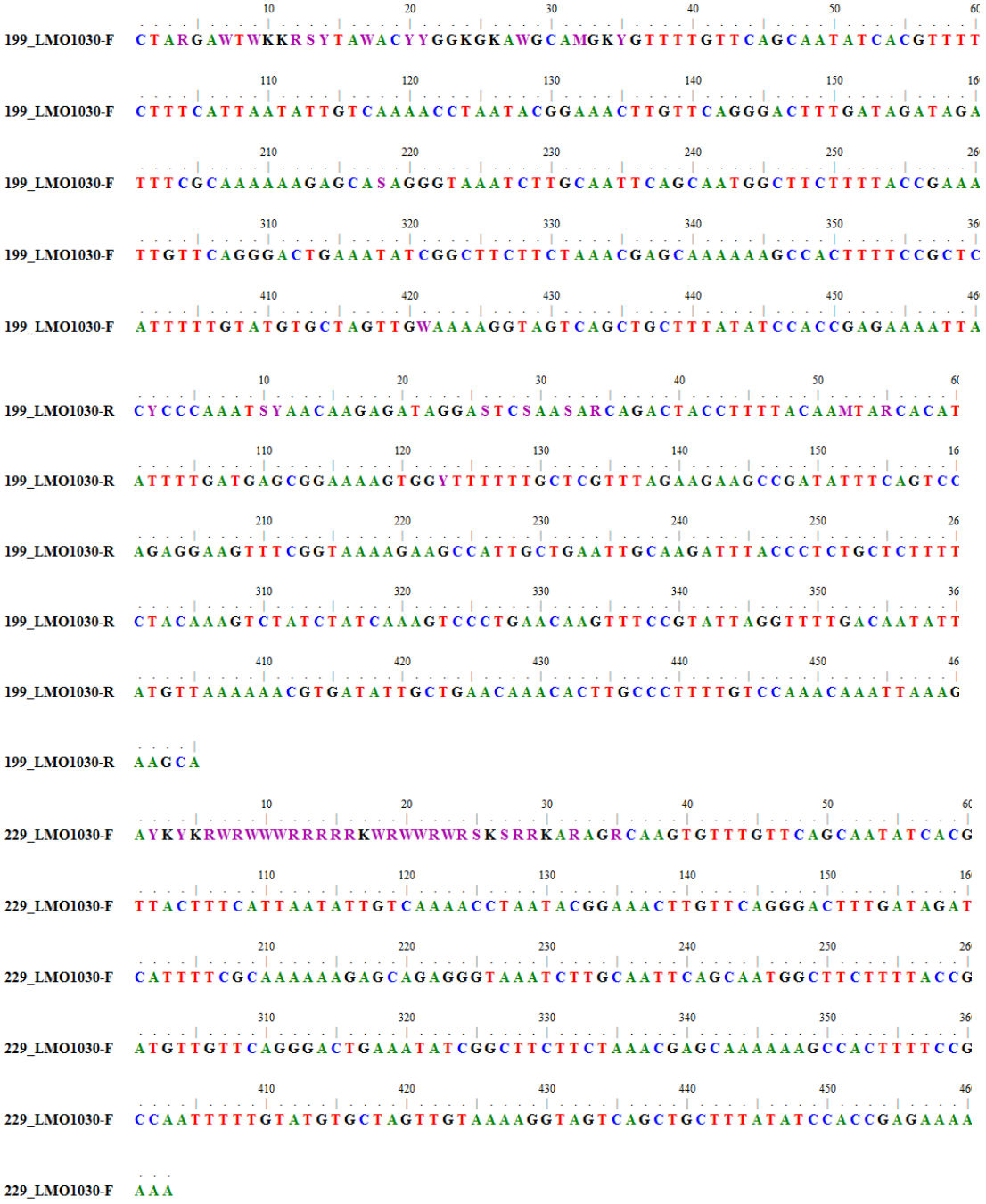
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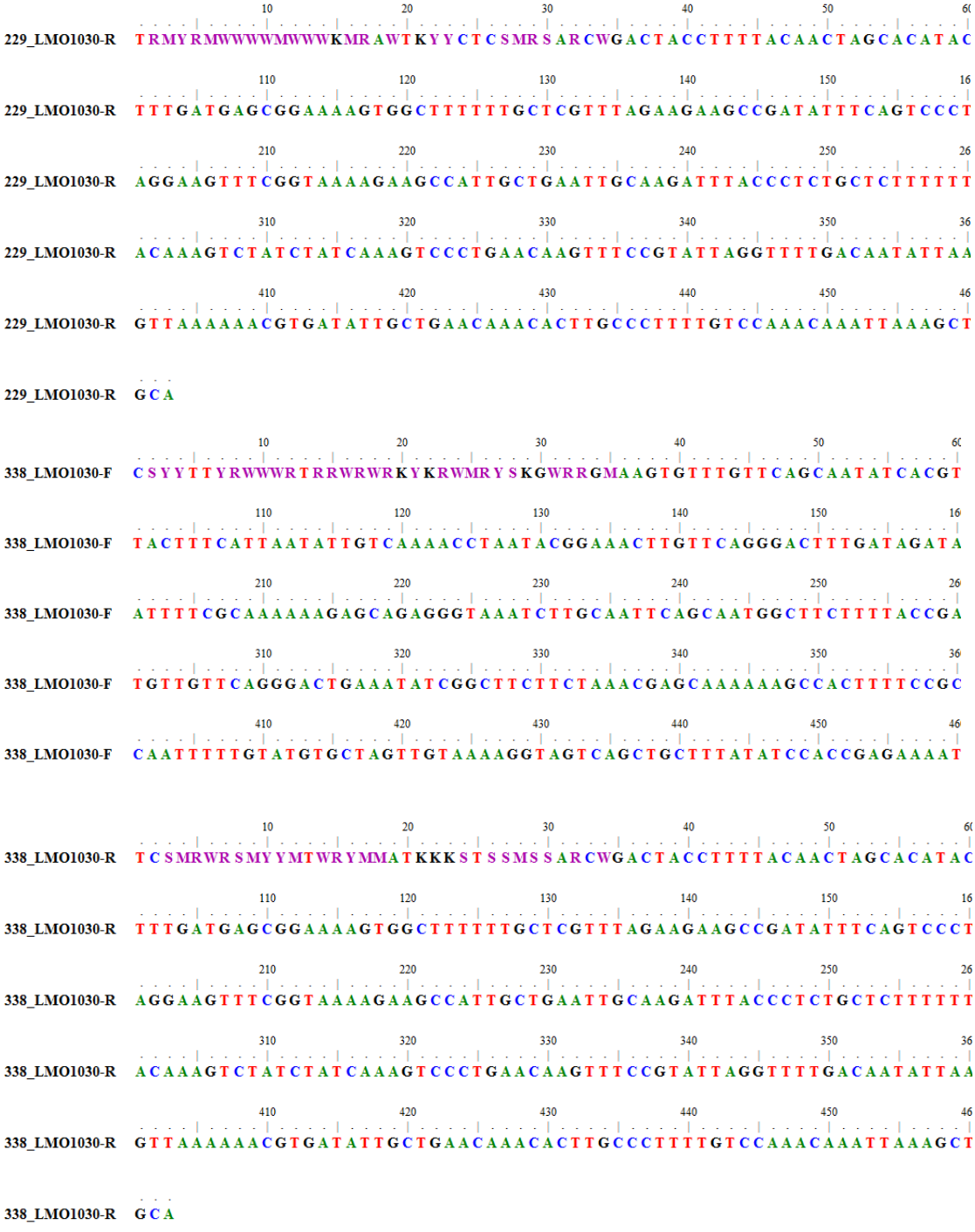
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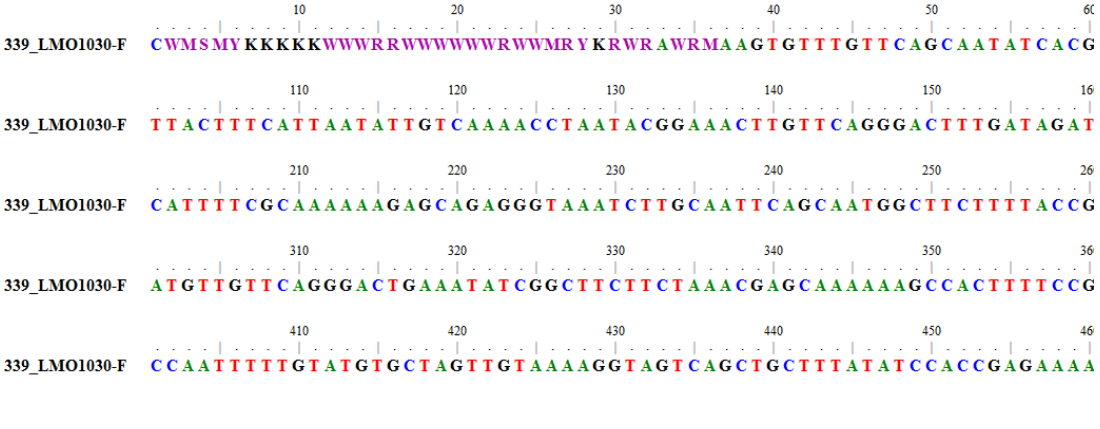
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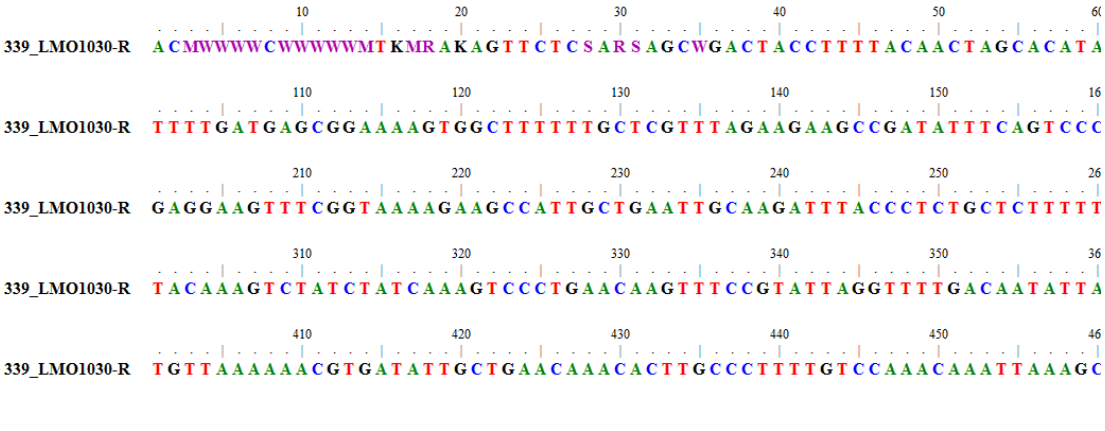
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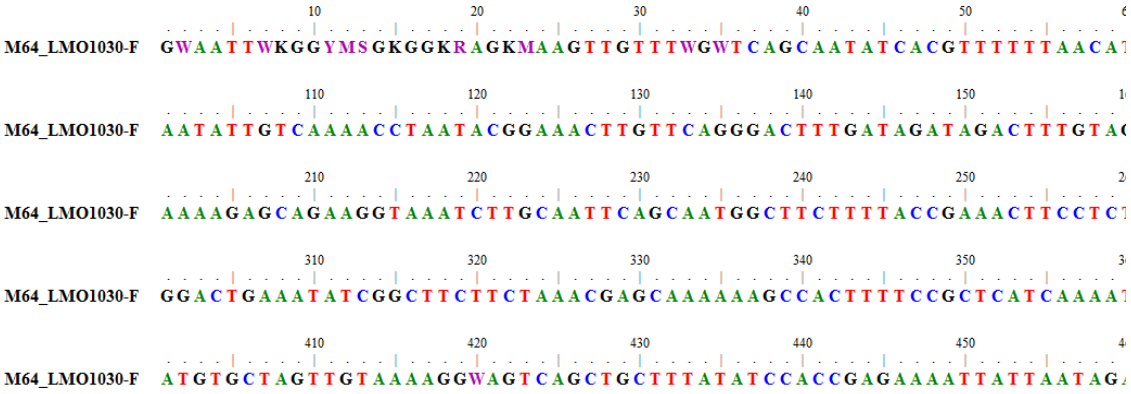
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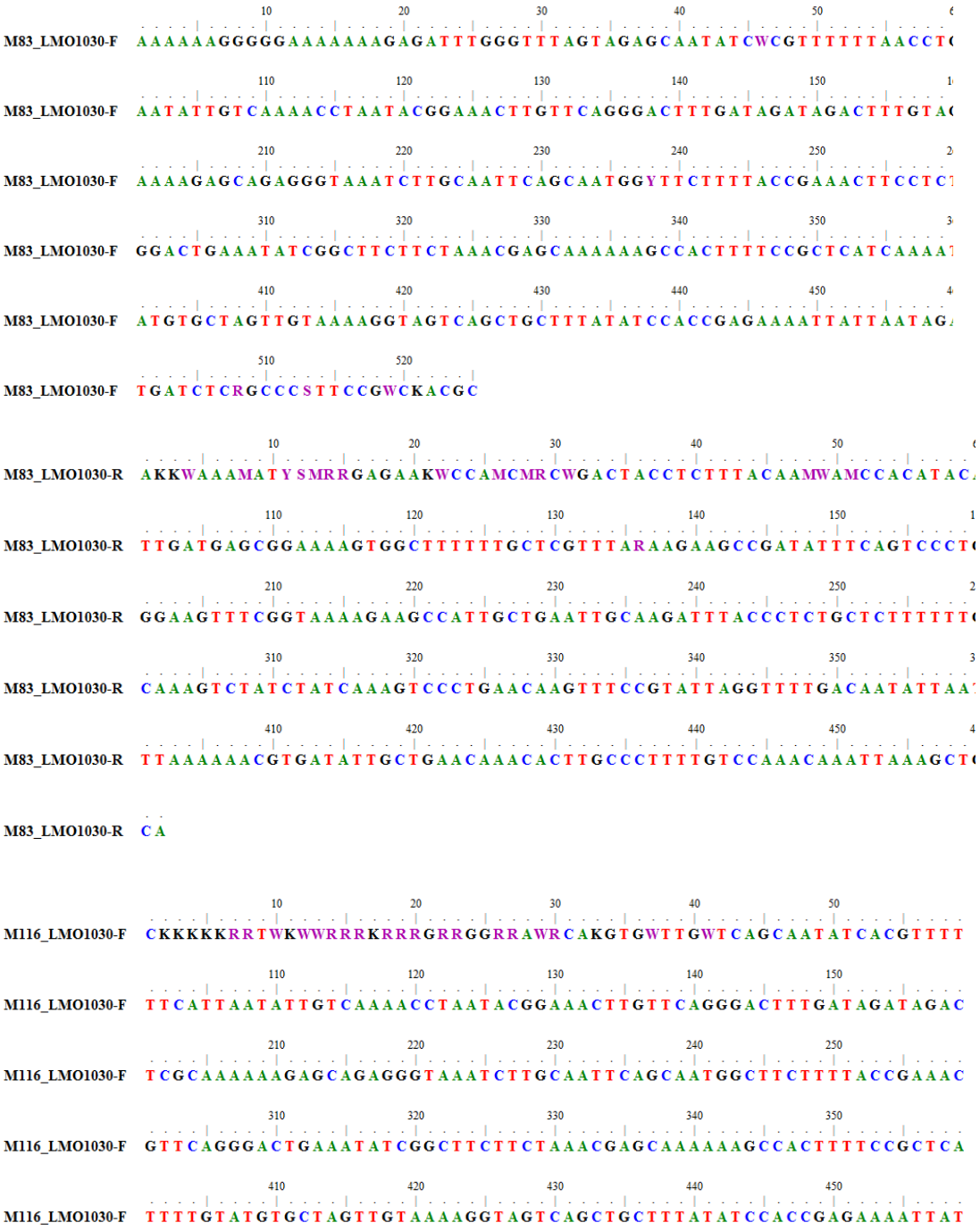
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M83_LMO1030-R CA
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APPENDIX V: Consensus Sequences

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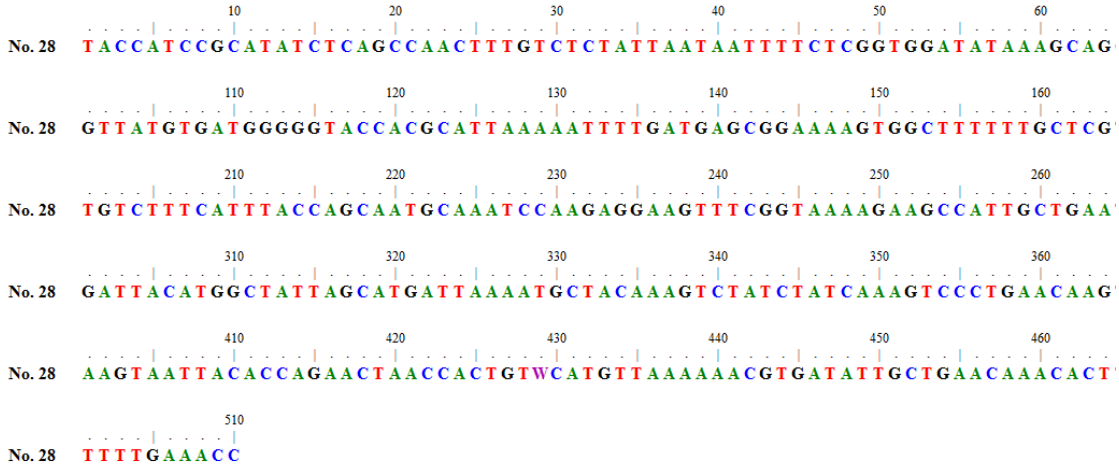

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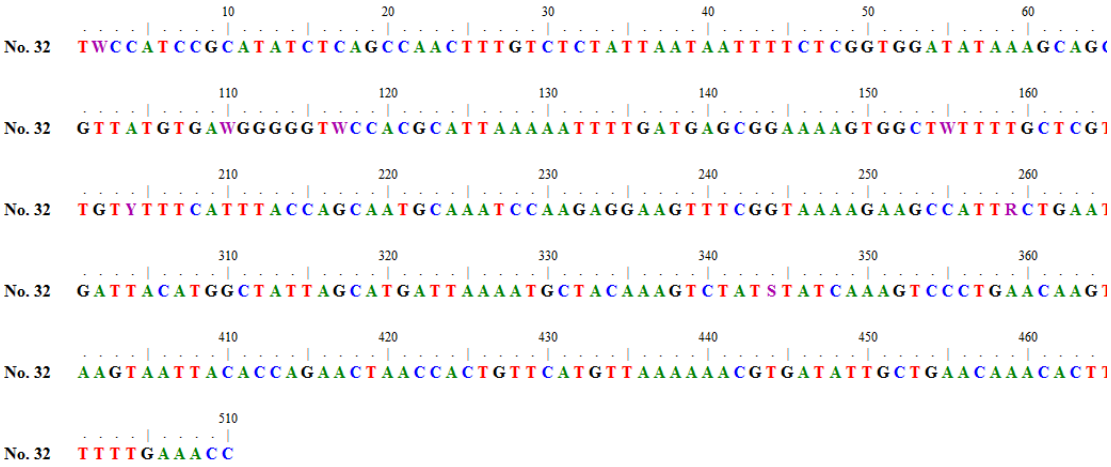

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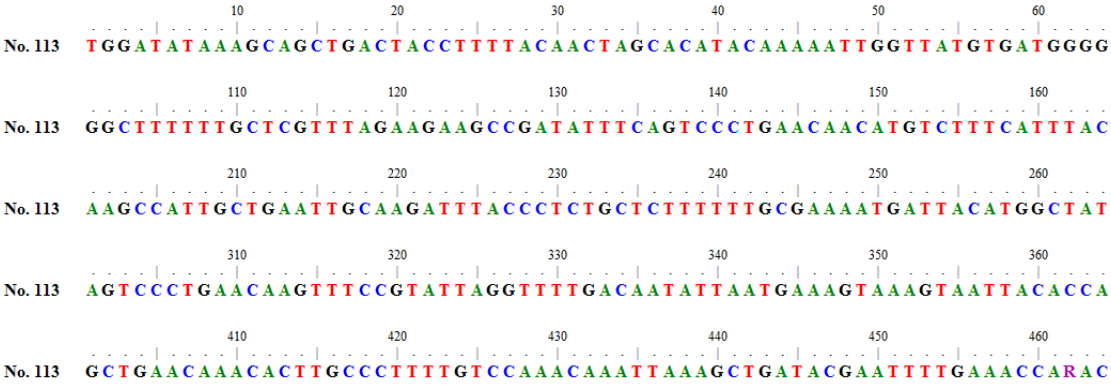

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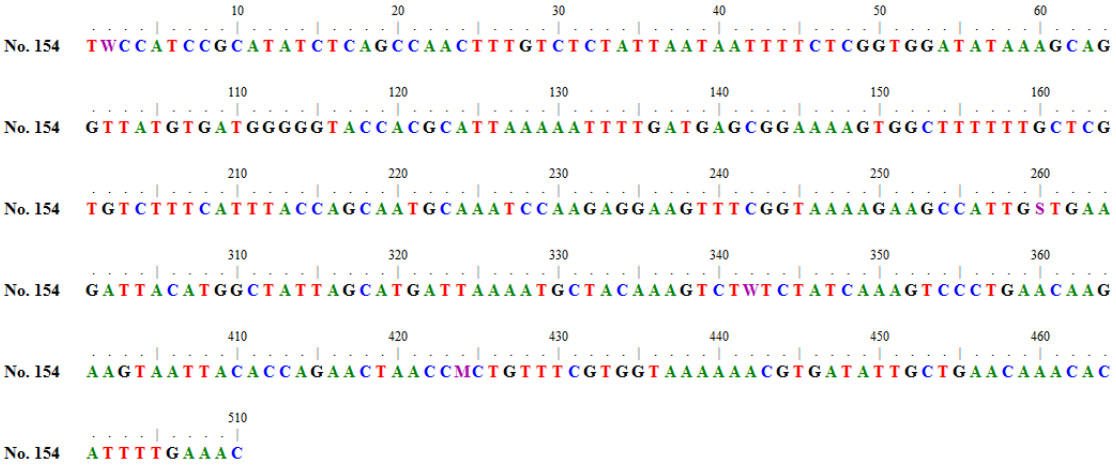

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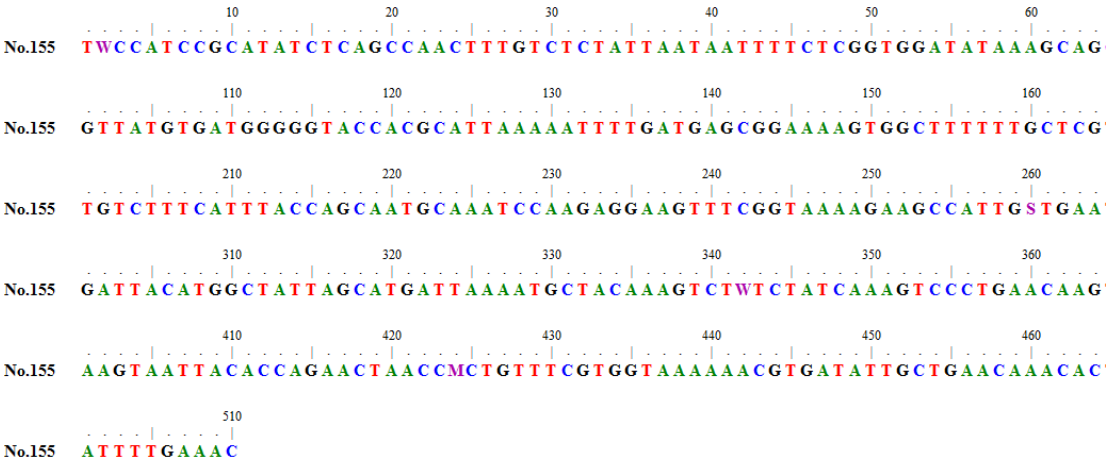

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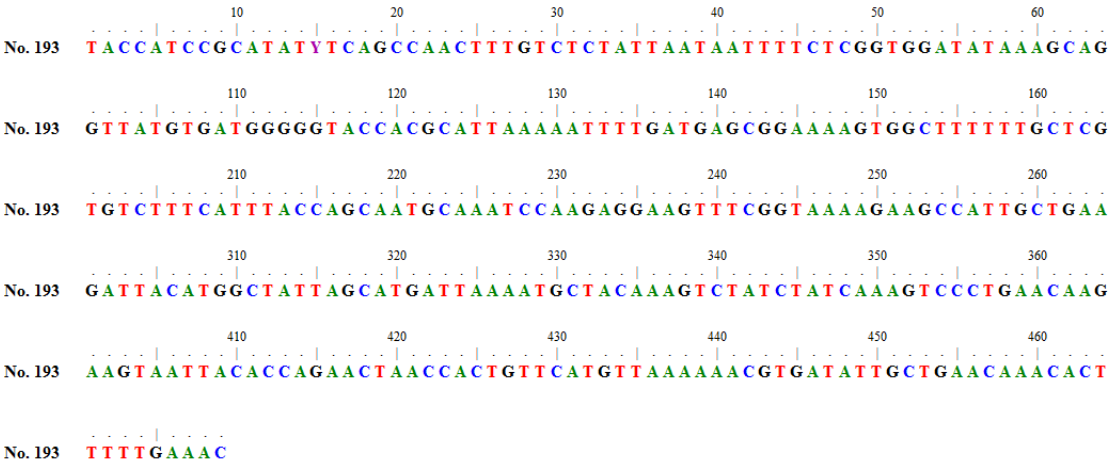

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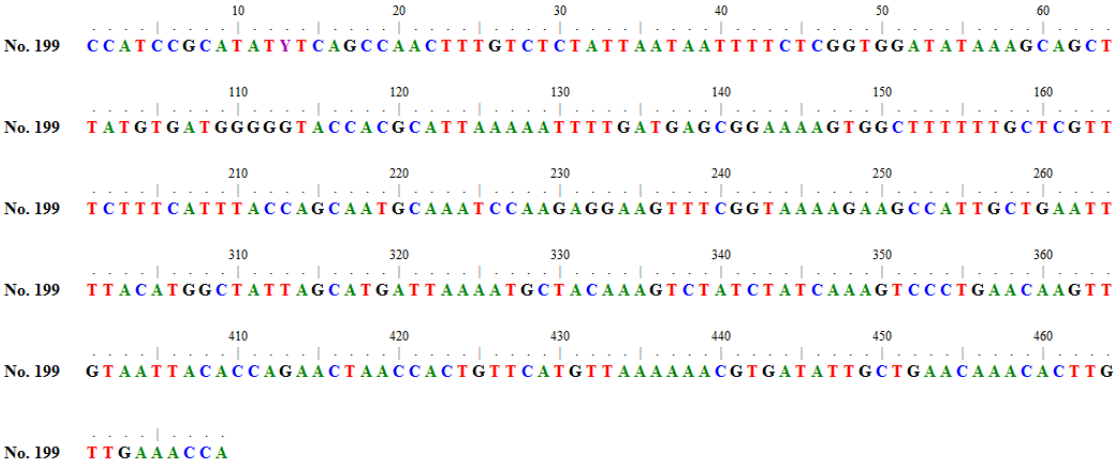

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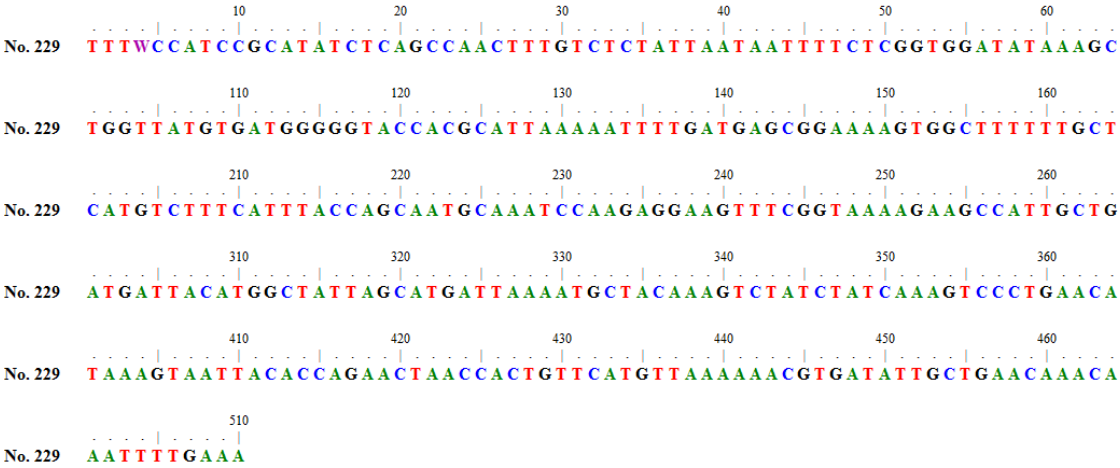

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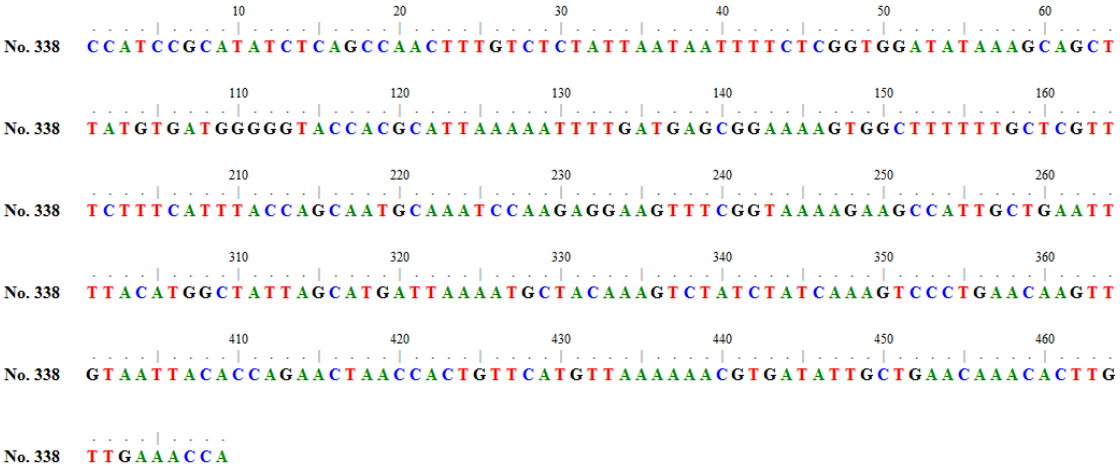

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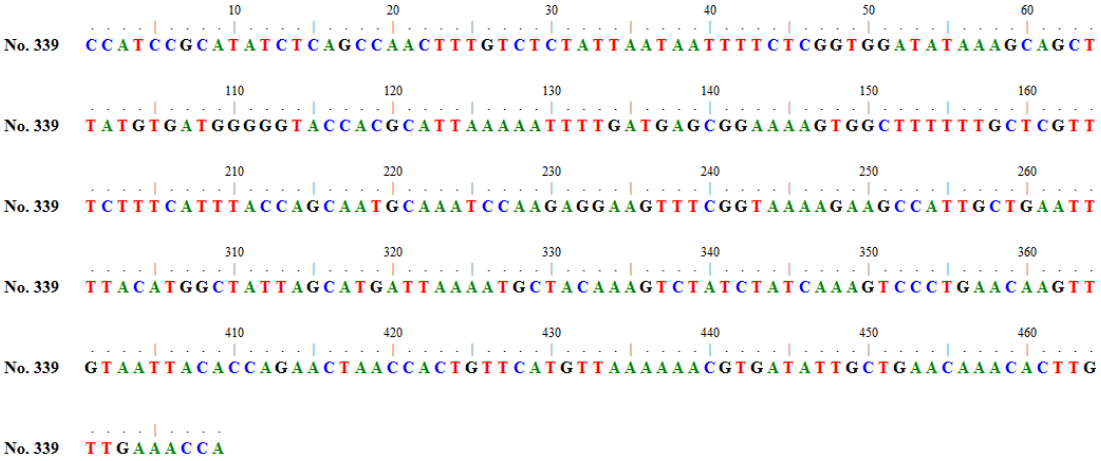

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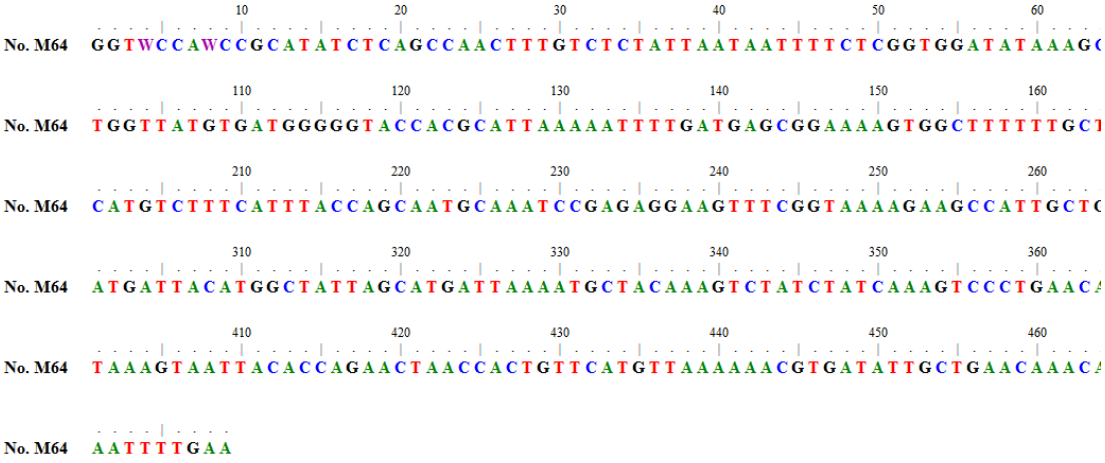

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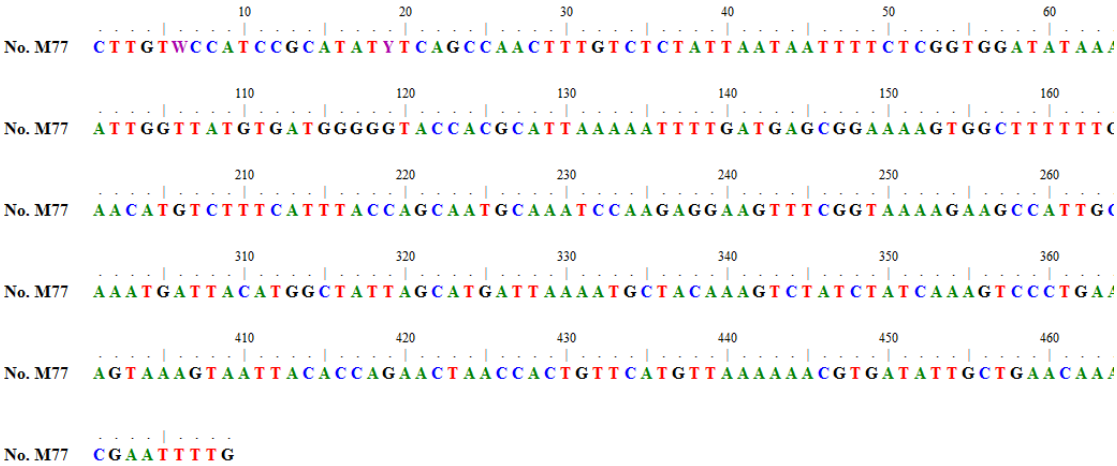

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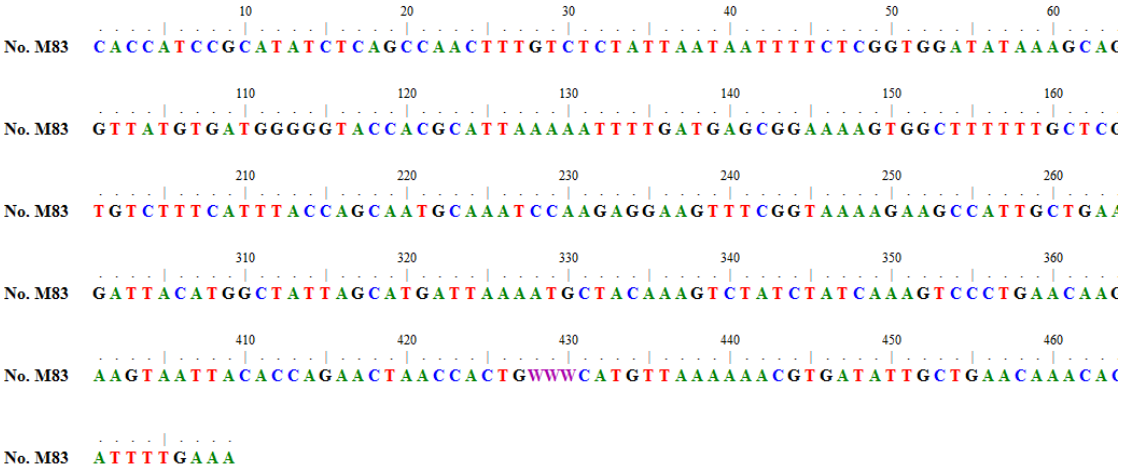

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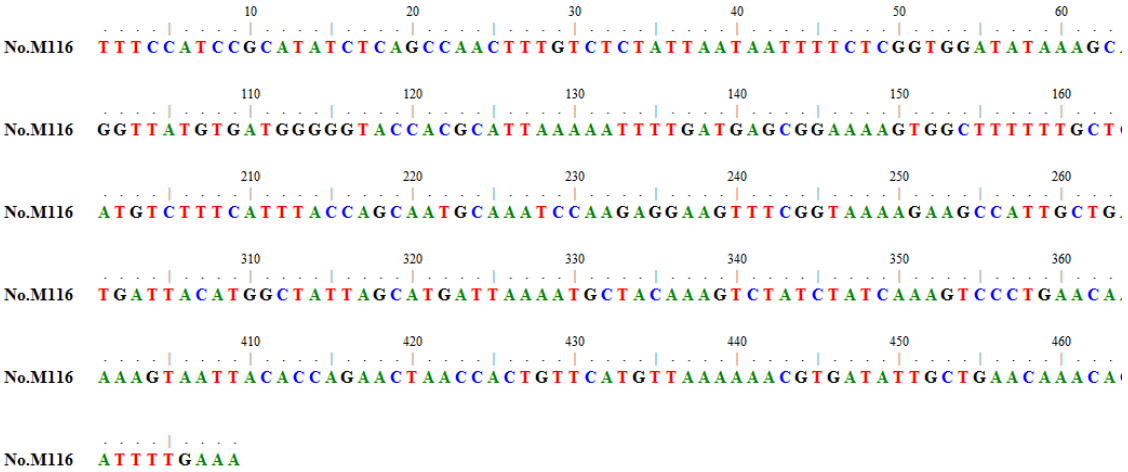

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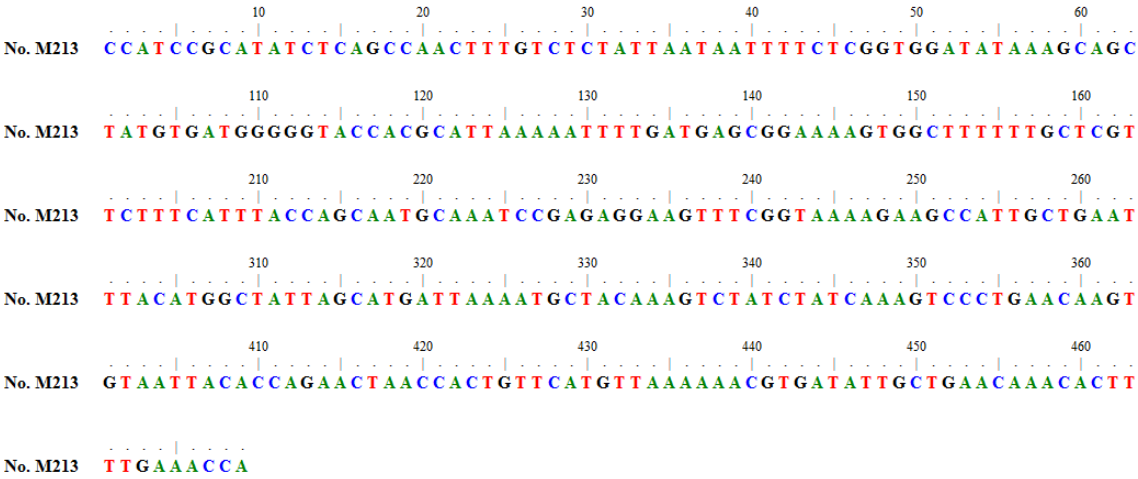

APPENDIX VI: Clinical and Laboratory Standards Institute (CLSI) breakpoints for *Staphylococcus spp*

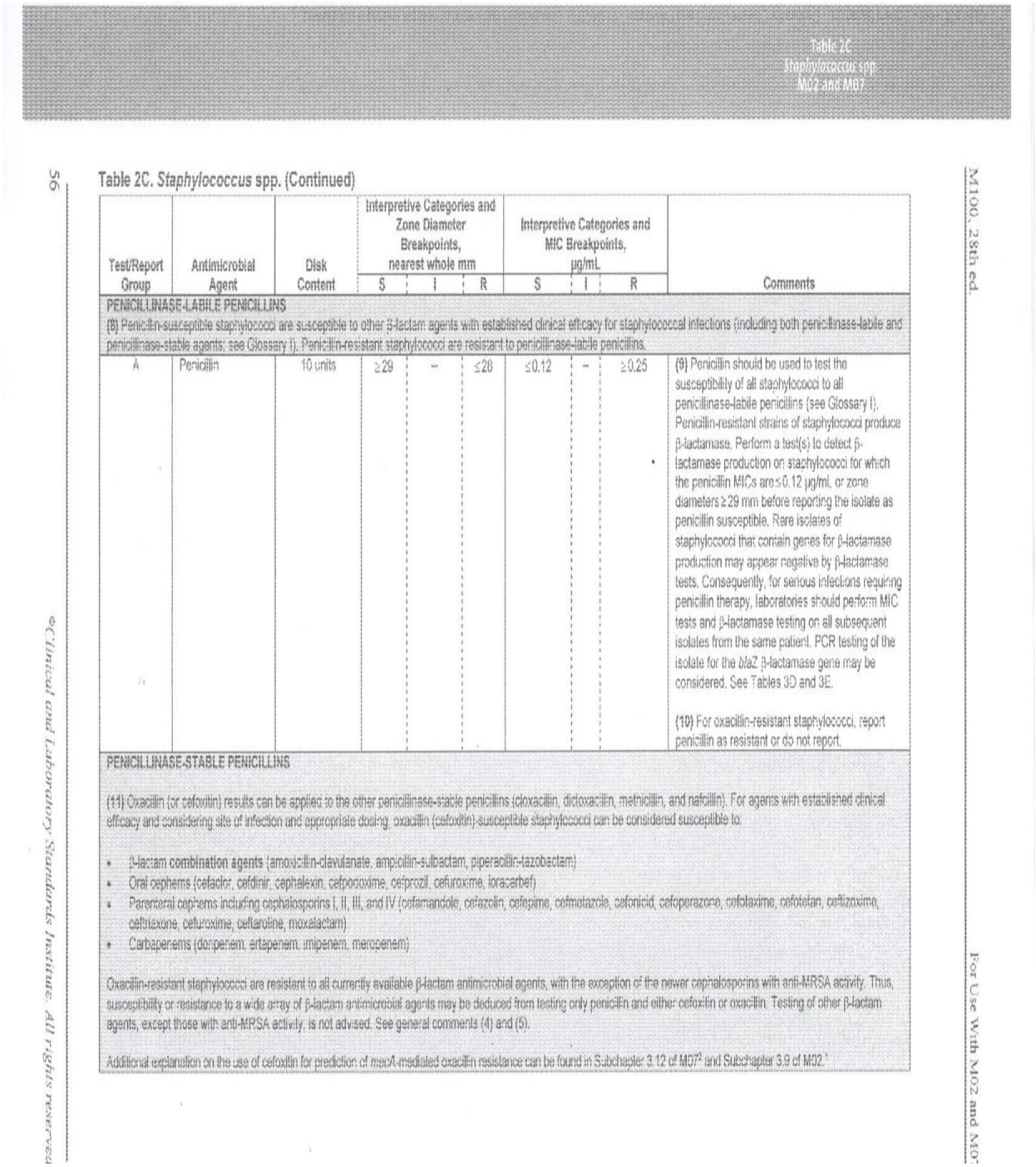

Table 2C
Staphylococcus spp.
M02 and M07

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Table 2C. *Staphylococcus* spp. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			Interpretive Categories and MIC Breakpoints, µg/mL			Comments
			S	I	R	S	I	R	
PENICILLINASE-LABILE PENICILLINS (8) Penicillin-susceptible staphylococci are susceptible to other β-lactam agents with established clinical efficacy for staphylococcal infections (including both penicillinase-labile and penicillinase-stable agents; see Glossary I). Penicillin-resistant staphylococci are resistant to penicillinase-labile penicillins.									
A	Penicillin	10 units	≥29	–	≤28	≤0.12	–	≥0.25	(9) Penicillin should be used to test the susceptibility of all staphylococci to all penicillinase-labile penicillins (see Glossary I). Penicillin-resistant strains of staphylococci produce β-lactamase. Perform a test(s) to detect β-lactamase production on staphylococci for which the penicillin MICs are ≤0.12 µg/mL or zone diameters ≥29 mm before reporting the isolate as penicillin susceptible. Rare isolates of staphylococci that contain genes for β-lactamase production may appear negative by β-lactamase tests. Consequently, for serious infections requiring penicillin therapy, laboratories should perform MIC tests and β-lactamase testing on all subsequent isolates from the same patient. PCR testing of the isolate for the *blaZ* β-lactamase gene may be considered. See Tables 3D and 3E. (10) For oxacillin-resistant staphylococci, report penicillin as resistant or do not report.
PENICILLINASE-STABLE PENICILLINS (11) Oxacillin (or cefoxitin) results can be applied to the other penicillinase-stable penicillins (cloxacillin, dicloxacillin, methicillin, and nafcillin). For agents with established clinical efficacy and considering site of infection and appropriate dosing, oxacillin (cefoxitin)-susceptible staphylococci can be considered susceptible to: • β-lactam combination agents (amoxicillin-clavulanate, ampicillin-sulbactam, piperacillin-tazobactam) • Oral cephems (cefaclor, cefdinir, cephalexin, cefpodoxime, cefprozil, cefuroxime, loracarbef) • Parenteral cephems including cephalosporins I, II, III, and IV (cefamandole, cefazolin, cefepime, cefmetazole, cefonicid, cefoperazone, cefotaxime, cefotetan, ceftizoxime, ceftriaxone, cefuroxime, ceftaroline, moxalactam) • Carbapenems (doripenem, ertapenem, imipenem, meropenem) Oxacillin-resistant staphylococci are resistant to all currently available β-lactam antimicrobial agents, with the exception of the newer cephalosporins with anti-MRSA activity. Thus, susceptibility or resistance to a wide array of β-lactam antimicrobial agents may be deduced from testing only penicillin and either cefoxitin or oxacillin. Testing of other β-lactam agents, except those with anti-MRSA activity, is not advised. See general comments (4) and (5). Additional explanation on the use of cefoxitin for prediction of *mecA*-mediated oxacillin resistance can be found in Subchapter 3.12 of M07[2] and Subchapter 3.9 of M02.[1]								

Table 2C. *Staphylococcus* spp. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			Interpretive Categories and MIC Breakpoints, µg/mL			Comments
			S	I	R	S	I	R	
AMINOGLYCOSIDES									
(24) For staphylococci that test susceptible, gentamicin is used only in combination with other active agents that test susceptible.									
C	Gentamicin	10 µg	≥15	13–14	≤12	≤4	8	≥16	
MACROLIDES									
(25) Not routinely reported on organisms isolated from the urinary tract.									
A	Azithromycin or	15 µg	≥18	14–17	≤13	≤2	4	≥8	
A	clarithromycin or	15 µg	≥18	14–17	≤13	≤2	4	≥8	
A	erythromycin	15 µg	≥23	14–22	≤13	≤0.5	1–4	≥8	
O	Telithromycin	15 µg	≥22	19–21	≤18	≤1	2	≥4	
O	Dirithromycin	15 µg	≥19	16–18	≤15	≤2	4	≥8	
TETRACYCLINES									
(26) Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline, minocycline, or both.									
B	Tetracycline	30 µg	≥19	15–18	≤14	≤4	8	≥16	
B	Doxycycline	30 µg	≥16	13–15	≤12	≤4	8	≥16	
B	Minocycline	30 µg	≥19	15–18	≤14	≤4	8	≥16	See comment (25).
FLUOROQUINOLONES									
(27) *Staphylococcus* spp. may develop resistance during prolonged therapy with quinolones. Therefore, isolates that are initially susceptible may become resistant within 3 to 4 days after initiation of therapy. Testing of repeat isolates may be warranted.									
C	Ciprofloxacin or	5 µg	≥21	16–20	≤15	≤1	2	≥4	
C	levofloxacin	5 µg	≥19	16–18	≤15	≤1	2	≥4	
C	Moxifloxacin	5 µg	≥24	21–23	≤20	≤0.5	1	≥2	
O	Enoxacin	10 µg	≥18	15–17	≤14	≤2	4	≥8	(28) For testing and reporting of urinary tract isolates only.
O	Gatifloxacin	5 µg	≥23	20–22	≤19	≤0.5	1	≥2	
O	Grepafloxacin	5 µg	≥18	15–17	≤14	≤1	2	≥4	
O	Lomefloxacin	10 µg	≥22	19–21	≤18	≤2	4	≥8	
O	Norfloxacin	10 µg	≥17	13–16	≤12	≤4	8	≥16	See comment (28).
O	Ofloxacin	5 µg	≥18	15–17	≤14	≤1	2	≥4	
O	Sparfloxacin	5 µg	≥19	16–18	≤15	≤0.5	1	≥2	
Inv.	Fleroxacin	5 µg	≥19	16–18	≤15	≤2	4	≥8	
NITROFURANTOINS									
U	Nitrofurantoin	300 µg	≥17	15–16	≤14	≤32	64	≥128	

Table 2C. *Staphylococcus* spp. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			Interpretive Categories and MIC Breakpoints, µg/mL			Comments
			S	I	R	S	I	R	
LINCOSAMIDES									
A	Clindamycin	2 µg	≥21	15–20	≤14	≤0.5	1–2	≥4	(29) Inducible clindamycin resistance can be detected by disk diffusion using the D-zone test or by broth microdilution (see Table 3G, Subchapter 3.9 in M02[1] and Subchapter 3.12 in M07[2]). See comment (25).
FOLATE PATHWAY ANTAGONISTS									
A	Trimethoprim-sulfamethoxazole	1.25/23.75 µg	≥16	11–15	≤10	≤2/38	--	≥4/76	
U	Sulfonamides	250 or 300 µg	≥17	13–16	≤12	≤256	--	≥512	(30) Sulfisoxazole can be used to represent any of the currently available sulfonamide preparations.
U	Trimethoprim	5 µg	≥16	11–15	≤10	≤8	--	≥16	
PHENICOLS									
C	Chloramphenicol	30 µg	≥18	13–17	≤12	≤8	16	≥32	See comment (25)
ANSAMYCINS									
B	Rifampin	5 µg	≥20	17–19	≤16	≤1	2	≥4	(31) *Rx:* Rifampin should not be used alone for antimicrobial therapy.
STREPTOGRAMINS									
C	Quinupristin-dalfopristin	15 µg	≥19	16–18	≤15	≤1	2	≥4	(32) For reporting against methicillin-susceptible *S. aureus*.
OXAZOLIDINONES									
B	Linezolid	30 µg	≥21	--	≤20	≤4	--	≥8	(33) When testing linezolid, disk diffusion zones should be examined using transmitted light. Organisms with resistant results by disk diffusion should be confirmed using an MIC method.
B	Tedizolid	--	--	--	--	≤0.5	1	≥2	See comment (17).

Abbreviations: ATCC®, American Type Culture Collection; BMHA, blood Mueller-Hinton agar; CAMHB, cation-adjusted Mueller-Hinton broth; CoNS, coagulase-negative staphylococci; I, intermediate; MHA, Mueller-Hinton agar; MIC, minimal inhibitory concentration; MRS, methicillin-resistant staphylococci; MRSA, methicillin-resistant *S. aureus*; PBP, penicillin-binding protein; PCR, polymerase chain reaction; QC, quality control; R, resistant; S, susceptible; UTI, urinary tract infection.