PREVALENCE AND ANTIBIOTIC SUSCEPTIBILITY OF *LISTERIA MONOCYTOGENES* ISOLATED FROM BROILER CHICKENS WITHIN DURBAN, SOUTH AFRICA

by

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Submitted in fulfilment of the academic
requirements for the degree of
Master of Science in the
School of Life Sciences,
University of KwaZulu-Natal
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December 2016

As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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ABSTRACT

The emergence of listeriosis as a disease due to food borne pathogen, *Listeria monocytogenes* (*L. monocytogenes*), has sparked the interest of many researchers since the 1980's. In this study, 148 broiler chicken samples were randomly collected from Durban in South Africa to examine the occurance and antibiotic susceptibility profile of *L. monocytogenes* and presumptive *Listeria* spp. Total sample size was 148 (n=148). The Polymerase Chain Reaction (PCR) was used to detect the presence of two virulence genes, *hlyA* and *inlB*. PCR was also used to detect for the presence of tetracycline resistant gene *tet*(S). Using the standard guidelines for Kirby-Bauer Disc Diffusion method, antibiotic susceptibility profiles were determined with the use of 10 antibiotics. Results demonstrated occurrence of 9% *L. monocytogenes* from the 148 samples tested. Fifty percent of the *L. monocytogenes* isolates were positive for the *hlyA* gene and the remaining 50% positive for the *inlB* gene. Presumptive *Listeria* spp. was observed at 8% from the total of 148 samples. Tetracycline resistance in *L. monocytogenes* isolates was reported at 71% and 69% for presumptive *Listeria* spp. The 10 antibiotics used for susceptibility testing embodied six classes of antibiotics i.e. Macrolides, Rifamycin, Glycopeptides, *β*-lactams, Chloramphenicols and Aminoglycosides. Highest resistance in *L. monocytogenes* isolates were observed for penicillin (93%), vancomycin and ampicillin (79%) and rifampicillin (64%). Presumptive *Listeria* spp. isolates showed highest resistance to ampicillin (48%), penicillin and vancomycin (39%), and rifampicillin and chloramphenicol (43%). The most susceptibility was observed for both *L. monocytogenes* and presumptive *Listeria* spp. isolates was the aminoglycoside antibiotic class. The *β*-lactams showed highest resistance overall in the *L. monocytogenes* isolates (55.5%) and 44% in the presumptive *Listeria spp*. The results obtained from this study emphasises the need for constant monitoring of poultry meat for the rise of antibiotic resistant *Listeria*. In conclusion, the occurrence of *L. monocytogenes* was low however the organism showed high resistance profiles for most antibiotics. This therefore implies that South Africa needs to implement a stricter regime of policies and controls when it comes to animal produced food products for human consumption.

Keywords: *Listeria monocytogenes*; antibiotic resistance; PCR; poultry; virulence

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville campus, from July 2014 to November 2016, under the supervision of Dr. O. T. Zishiri.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION 1 – PLAGIARISM

I, Shrinav Rai Dawlat, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

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DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1: (This study is still under review for publication)

Dawlat, S. R. and Zishiri, O.T. (2016). Prevalence and Antibiotic Susceptibility of *Listeria Monocytogenes* Isolated From Broiler Chickens Within Durban, South Africa.

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ACKNOWLEDGEMENTS

I would to give thanks to my supervisor, Dr. O. T. Zishiri, for his constant support, patience and valued input in both an academic and personal capacity. His guidance and belief in my ability has made me achieve new heights and his motivational lectures have kept me going.

I am grateful to my parents who have supported me emotionally and financially throughout my studies as well as providing the much needed get-up-and-go motivation.

I would like to express my gratitude towards my peers, all honours and masters students within the Genetics Department of the University of KwaZulu- Natal. You all have provided me with an academic sound board as well as a great studying environment.

Finally, I would like to give my heart felt appreciation and thanks to my girlfriend, Candice Ramoudh. You have been my pillar of strength throughout my studies and have consistently reminded me of what I am going to achieve in life. It is due to your unconditional love and support that kept me burning the midnight oil.

CHAPTER 1

1 GENERAL INTRODUCTION

Poultry farming in South Africa serves as a major source of income to the economy as both broilers and eggs are exported on a large scale to neighboring countries. In 2015, a total of 1925 tons fertilized chicken eggs, 226.6 tons of fertile eggs other than chicken and ostrich, 1.5 tons of ostrich eggs and 2015 tons of egg products were exported (South African Poultry Association, 2015). Poultry exports in 2014 amounted to 66 355 tons which was a 162% increase in export from 2013. From these exports, 93% were broiler products which generated 36 million rand (South African Poultry Association, 2015). One of the major concerns within poultry farming is disease and infection of poultry due to unhygienic or crowded poultry houses. The world-wide commercial poultry industry is well-developed and is the largest supplier of animal protein in the form of meat and eggs. Its significance is even greater in developing countries where poultry are relatively cheap and can be kept in a small area, usually providing both protein and some income for a family (Law and Payne, 1996). Food-borne illnesses account for the majority of illnesses reported in most developed countries. According to a World Health Organization (WHO) report of 2005, there were 1,8 million deaths from diarrheal diseases caused by contaminated food. It has also been reported that 30% of populations in industrialized countries succumb to food-borne illnesses every year. Food-borne disease is a common public health problem worldwide, but is generally under-reported and poorly investigated in South Africa and southern Africa at large (Miliotis and Bier., 2003).

Considering the estimated under-reporting of food-borne illnesses in South Africa, the estimated medical costs, productivity losses and value of premature deaths due to diseases caused by five food-borne pathogens (*Campylobacter*, non-typhi *Salmonella*, *Escherichia. coli* O157, *E. coli* non-O157 STEC and *Listeria monocytogenes*) runs into billions of Rands per year (Miliotis and Bier., 2003). In chicken meat samples, the occurrence of *E. coli* contamination was 60 %, 100 %, 44.4 %, 100 % and 80 % in five different regions of North West Province (Mabote *et al*., 2011). Futhermore, the following species were also detected: *Listeria* spp. (22%), *Enterobacter* spp. (18%), *Staphylococcus aureus* (3.2%). Seventeen food-borne pathogens were recovered from ready-to-eat (RTE) Foods from Roadside cafeterias and retail outlets in Alice, Eastern Cape Province (Nyenje *et al*., 2012).

Listeriosis was known in animals however, when the spread of infection moved from animals to a food borne pathogen in humans during the 1980's, it sparked interest and intensive research into the genus *Listeria*. Taxonomically, the genus *Listeriae* comprises of nineteen *Listeria* species; *L. monocytogenes, L. ivanovii, L. welshimeri, L. grayi, L. seeligeri, L. innocua, L. marthii, L. fleischmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. rocourtiae, L. weihenstephanesis, L. grandensis, L. riparia, L. booriae, L. denitrificans and L. murrayi* as described by Orsi and Weidmann. (2016). Of these 19 species only *L. monocytogenes* and *L. ivanovii* are pathogenic.

Characterization of *L. monocytogenes* was first described by Murray *et al*. (1926) and Pirie (1927). *Listeria* is a small rod shaped gram positive bacterium measuring 1-2µm in length and 0.5µm wide. *Listeria* is often found parallel to each other forming palisades while growth occurs between 3°C and 45°C however, the optimal range is between 30°C to 37°C (Duffy *et al*., 1994). It is best grown in anaerobic or micro-anaerobic conditions, pH ranges between 5.6 to a high of 9.6. Colonies appear smooth, silky and white but when illuminated with obliquely transmitted light, the colonies may appear as a blue/green sheen. When grown at 37°C there is no motility however, when incubated at room temperature with motile organisms, a typical tumbling motion is observed (Duffy *et al*., 1994).

Biochemically inactive, *Listeria* produce catalase, positive in the Vogues-Proskauer reaction and hydrolyse aesculin (Low and Donachie, 1997). They are indole and oxidase negative, do not hydrolyse urea or reduce nitrates and no liquefaction of gelatin. Important in the identification of *Listeria* species is the appearance of haemolysis. *Listeria monocytogenes* inoculated together with *Staphyloccocus aureus* shows the haemolysis known as the CAMP effect. Typing was done using experiments that involved hyper-immune serum and early seriological studies. In the 1940's studies were conducted on the somatic "O" and flagellar "H" antigens to describe four possible serotypes. Common problems with serotyping are the inability to differentiation between species with a number of serovars belonging to different species of *Listeria* (Low and Donachie, 1997).

L. monocytogenes is widely distributed in nature and has been isolated from a wide array of food products. The organism is considered hazardous in the food industry due to its ability to grow in gas or vacuum-packaged products at refrigeration temperatures (Duffy *et al*., 1994), low water activity (Nolan *et al*., 1992) as well as low pH (Buchanan *et al*., 1993) and all these measures are important

in the control of food pathogens. *L. monocytogenes* is also problematic due to its resistance to antibiotics. The first multi-resistant strain of *L. monocytogenes* was isolated in France in 1988 (Poyart -Salmeron *et al*., 1990), thereafter, *L. monocytogenes* strains resistant to one or more antibiotics have since been isolated (Albuin *et al*., 1994; Charpentier *et al*., 1995). It has been shown by various studies that listeriosis is a food-mediated illness (Embarek, 1994; Slutsker and Schuchat, 1999). A wide range of foods such as salads, seafood, meat, and dairy have been implicated in listeriosis (Bell and Kyriakides, 1998), which follows the oral ingestion of the contaminated food (Finlay, 2001). Serotyping has been used extensively to characterize *L. monocytogenes* (Wiedmann, 1993; Wagner and Allerberger, 2003). Thirteen *L. monocytogenes* serotypes (serovars) have been characterized by using specific and standardized antisera (Seeliger and Hohne, 1979). Although most clinical isolates belong to serovars 1/2a, 1/2b, and 4b, the majority of strains which have caused large 5 outbreaks were serovar 4b (Kathariou, 2000), and serovar 1/2a (Jacquet *et al*., 2002; Zhang and Knabel, 2005). Serovar identification by serological tests has remained popular. However, numerous molecular biology methods such as multiplex PCR (Doumith *et al*., 2004) have come to the fore in the characterization of *L. monocytogenes* serotypes.

Listeriae are non-spore forming, rod shaped, gram positive bacteria. Due to *Listeria monocytogenes* being an intracellular food borne pathogen which may be lethal to animals and humans, the presence of virulence genes has led intensive studies during the 1980's (Osaili *et al*., 2011). It is important to note that *Listeria ivanovii* is pathogenic in animals; however, there are few reported cases of infection in humans. All *Listeria* from the genus inhabit a wide and diverse environment and are physiologically similar. Many phylogenic studies have been conducted to compare the genomes of the pathogenic *L. monocytogenes* and *L. ivanovii* to their non-pathogenic counterparts (Osaili *et al*., 2011).

Listeria monocytogenes causes a very serious illness known as listeriosis. Individuals who are particularly susceptible to this condition are those who are immune-compromised (as in HIV/AIDS infection), pregnant women, newborn babies, and the elderly (Farber and Peterkin, 1991; McLauchlin *et al*., 2004). Although the incidence of listerosis is low, what is significant is the very high fatalities ranging from 20 to 30% have been reported (Mead *et al*., 1999). Even though a recent study (Manani *et al*., 2006) reported the occurrence of *L. monocytogenes* in frozen vegetables in this country, there is little data on the occurrence of this pathogen in South Africa. The current body of knowledge is available with worldwide studies however; there is a paucity of published information

on the occurrence of *Listeria* spp. and the associated antimicrobial and virulence genes as well as antimicrobial susceptibility profiles in South Africa. Against this background, this study aimed to study the occurrence and antibiotic susceptibility of *L. monocytogenes* isolated from broiler chickens within Durban, South Africa.

1.1 Aims

i. To identify the occurrence of *L. monocytogenes* in local bred chickens within Durban, KwaZulu-Natal.
ii. To identify to virulence genes present *L. monocytogenes* obtained from the chickens in Durban, KwaZulu- Natal.
iii. To test positive *Listeria* samples against a variety of antibiotics in order to create an antibiotic resistance profile.

1.2 Research Objectives

i. Using selective agars and broths to grow *Listeria* species obtained from chickens around Durban, KwaZulu- Natal.
ii. Using Polymerase Chain Reaction (PCR), amplify virulence genes for the identification of *L. monocytogenes* as well as to identify virulence within these samples.
iii. Using the PCR to amplify antibiotic resistance genes in positive *Listeria* species obtained from samples.
iv. Using the Kirby-Bauer Disc Diffusion method to test *Listeria* species against a variety of antibiotics for antibiotic resistant profiling.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Introduction

Food-borne illnesses account for the majority of illnesses reported in most developed countries. According to a World Health Organization (WHO) report of 2005, there were 1,8 million deaths from diarrheal diseases caused by contaminated food globally. It has also been reported that 30% of populations in industrialized countries succumb to food-borne illnesses every year (Miliotis and Bier., 2003). Food-borne disease is a common public health problem worldwide, but is generally under-reported and poorly investigated in South Africa and southern Africa at large (Miliotis and Bier., 2003).

A report done by Alexandra Sifferlin for Time magazine covers a *Listeria* outbreak that occurred in May 2016 in the US. More than 360 frozen food products were recalled from shelves and according to the Center for Disease and Control (CDC), *Listeria* outbreaks results in 1 600 Americans being sick each year. *Listeria* outbreaks have also been recorded as the third leading cause of death due to food poisoning in the US. During the course of 2011 to 2016, the CDC has released reports on *Listeria* contamination in milk, refrigerated salads, cheese, ice creams and apples with outbreaks increasing over these years. This section therefore reviews *L. monocytogenes*, the disease caused by infection with *L. monocytogenes* as well as outbreaks caused by this organism, antibiotic resistance developed by this organism and current controls to manage listeriosis and resistance in *L. monocytogenes*.

2.1.1 The Global Poultry Industry

The poultry industry is based on the production of two types of products, namely eggs and meat. There are many interconnections between the egg and poultry meat industries. A few breeding enterprises produce day-old chicks of both egg and meat types. The layer type is used to produce table eggs while the meat type produces broilers. The two industries may operate from a common base of layer or broiler breeders, stock-feed mills, equipment and pharmaceutical suppliers (Henry and Rothwell, 1995).

Poultry is one of the world's fastest growing sources of meat, representing 31.5% of all meat produced in 2007 (Global Poultry Trends, 2007). The modern broiler industry started in the 1930s, when flock size was seldom greater than a few hundred birds. By the 1950s, the flock size had increased to a few thousand and by the 1980s many broiler houses had a capacity of 100 000 or more (Hubbert *et al*., 1996). Globally, there are about 60 billion broilers at any one time, of which 26.6 percent are in the USA, 16.9 percent in the People's Republic of China, 16.4 percent in Brazil and 12.3 percent in the European Union (USDA-FAS, 2007). China consumes almost 17.5 percent of global production compared to the USA which consumes 23.5 percent and the EU which consumes 12.3%. (USDA-FAS, 2007).

2.2 *Listeria* and Listeriosis

Figure 1, shows a phylogenetic tree constructed based of concatenated amino acid sequences of 352 single gene copies within all *Listeria* species. This tree shows new proposed genus of *Listeria* according to the clades as described by Orsi and Weidmann modified from Weller *et al*., 2015 and Chiara *et al*., 2015. Each clade respresents the genus *Listeria* which includes the following species; *L. monocytogenes, L. marthii, L. innocua, L. welshimeri, L. ivanovii* and *L. seeligeri*. The second clade represents the proposed genus Murraya which includes the species; *L. grayi* DSM 20601 and *L. grayi* ATCC 25401. The third clade is the proposed genus *Mesolisteria* which includes the species; *L. fleischmanii* subsp. *fleischmanii* LU2006_1, *L. fleischmanii* subsp. *fleischmanii* TTU M1-001, *L. floridensis* FSL S10-1187 and *L. aquatica* FSL S10-1088. The fourth clade is the proposed genus *Paenilisteria* which includes the species; *L. newyorkensis* FSL A5-0209, *L. newyorkensis* FSL M6-0630, *L. cornellensis* TTU A1-0210, *L. weihenstephanesis* DSM 24699, *L. grandensis* TTU A1-0212, *L. riparia* FSL S10-1204 and *L. booriae* FSL A5-0281. The tree is 96% reliable. The proposed new genus for *Murraya* is the isolation of *L. grayi*, all species which cannot grow below 4°C are grouped into the genus *Mesolisteria* and finally the seven new species are grouped into the genus *Paenilisteria.*

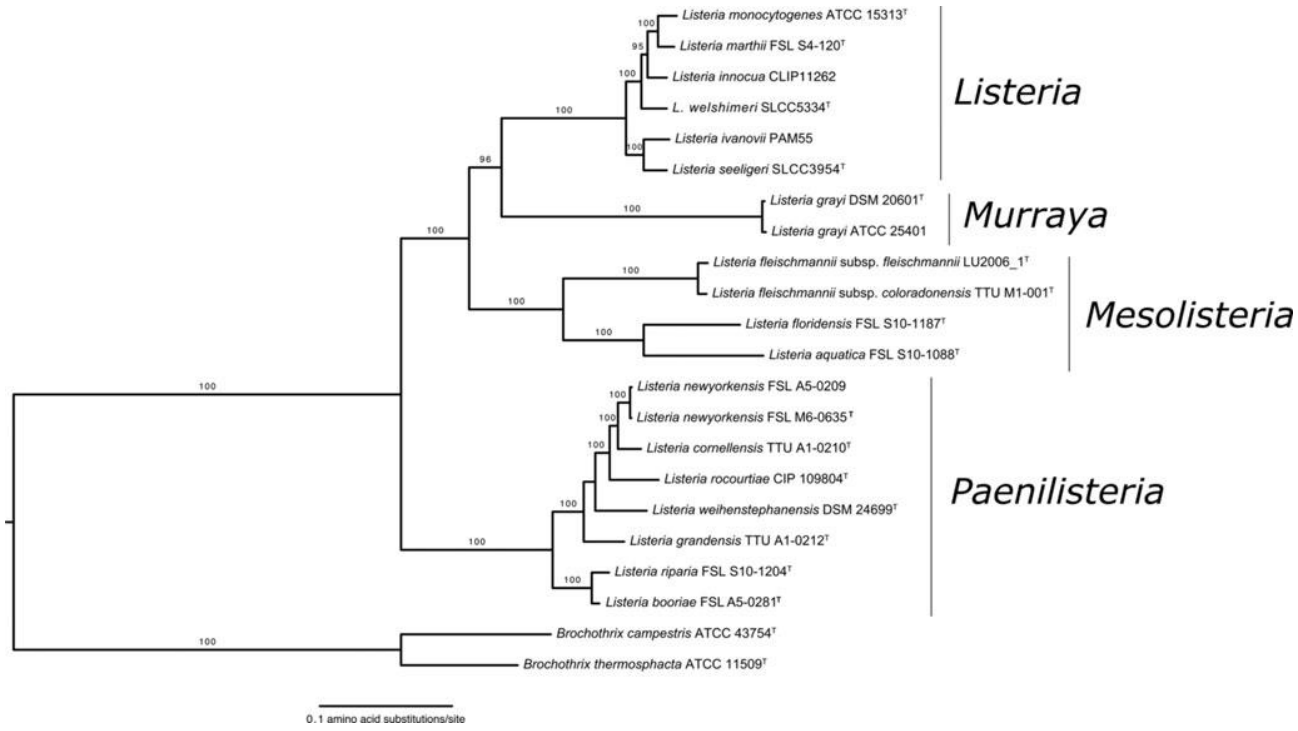

Figure 1: Maximum Likelihood phylogentic tree adapted from Orsi and Weidmann., 2016 showing newly proposed genus clades for the seventeen *Listeria* species. Tree constructed using 250 bootstraps replicates with boostrap values >70% shown on branches and bar represents 0.1 amino acid substitutions per site.

A wide range of foods such as salads, seafood, meat, and dairy have been implicated in listeriosis (Bell and Kyriakides, 1998; Schlech, 2000), which follows the oral ingestion of the contaminated food (Finlay, 2001). Serotyping has been used extensively to characterize *L. monocytogenes* (Wiedmann, 1993; Wagner and Allerberger, 2003). Thirteen *L. monocytogenes* serotypes (serovars) have been characterized by using specific and standardized antisera (Seeliger and Langer, 1979). Although most clinical isolates belong to serovars 1/2a, 1/2b, and 4b, the majority of strains which have caused large 5 outbreaks were serovar 4b (Kathariou, 2000), and serovar 1/2a (Jacquet *et al*., 2002; Zhang and Knabel, 2005). Serovar identification by serological tests has remained popular. However, numerous molecular biology methods such as multiplex PCR (Doumith *et al*., 2004) have

come to the fore in the characterization of *L. monocytogenes* serotypes. Even though a study (Manani *et al*., 2006) reported the occurrence of *L. monocytogenes* in frozen vegetables in this country, there is little data on the occurrence of this pathogen in South Africa.

2.3 Growth conditions

Listeria monocytogenes is a Gram positive, non-spore forming, facultative anaerobe, intracellular pathogen (short rod) which grows between -4 and 50 °C and a pH range of 4.39 to 9.40. *Listeria monocytogenes* can also multiply in blood, cerebrospinal fluid, monocytes, macrophages, leucocytes and T-cells at body temperature of 37 °C (Farber *et al.,* 1991).

Listeria has been isolated sporadically from a wide variety of sources and listeriosis outbreaks that have occurred in the early 80's have highlighted contaminated food as the main source of transmission (Farber *et al*., 1991). A variety of RTE food products, such as, frozen or raw vegetables, milk and milk products, meat and meat products and seafood support the growth of organism. Usually the presence of *Listeria* species in food is thought to be an indicator of poor hygiene (Manani *et al.,* 2006).

Twenty three Serotypes of *Listeria monocytogenes* strains are serotyped according to variation in the somatic (O) and flagellar (H) antigens (Seeliger and Hohne, 1979). Although more than 13 serotypes of *L. monocytogenes* have been described, only three serotypes (1/2a, 1/2b, and 4b) are usually predominant in clinical cases (Tappero *et al*., 1995). Interestingly, although serotype 1/2a is most frequently isolated from food, it is serotype 4b which causes the majority of human epidemics (Gilot, 1996).

2.4 *Listeria* disease

Ingestion of food contaminated with *L. monocytogenes* can result in listeriosis; a severe infectious disease characterized by meningoencephalitis, abortion, septicemia and a high fatality rate of 30% was traced to *Listeria* (Cocolin *et al*., 2002). Listeriosis predominantly affects certain risk groups including pregnant women, newborn babies, elderly people and immune-compromised patients including HIV positive patients. Most healthy individuals experience flu-like symptoms and those at high risk include cancer patients, individuals taking drugs that affect the body's immune system,

alcoholics, pregnant women, persons with low stomach acidity and individuals with HIV/AIDS (Rodriguez – Lazaro *et al.,* 2004).

This organism causes listeriosis, clinically defined when the organism is isolated from blood, cerebrospinal fluid and even in placenta and fetus in abortion cases. The manifestation of listeriosis include septicemia, meningitis (meningoencephalitis), encephalitis and intrauterine or cervical infections in pregnant women which may result in spontaneous abortions (2^{nd} or 3^{rd} trimester) or still birth. The onset of the aforementioned disorders is usually preceded by influenza-like symptoms including persistent fever followed by nausea, vomiting and diarrhea, particularly in patients who use antacid or cimetidine (Tominaga *et al.,* 2006).

The onset time to serious forms of listeriosis ranges from a few days to 3 weeks. The onset time to gastrointestinal symptoms is greater than 12 hours. *L. monocytogenes* invades the gastrointestinal epithelium. Once the bacterium enters the host's monocytes, macrophages or polymorphonuclear leukocytes, it is blood-borne (septicemia) and can grow in body cells (Sebelius *et al.,* 1999). *Listeria monocytogenes* produces an exotoxin listeriolysin (LLO) which is a key agent in human neutrophil activation. The stimulation of these phagocytes, however, requires additional listerial virulence factors of which PIcA may play a prominent role (Sibelius *et al.,* 1999).

2.5 Determinants of Virulence in *Listeria*

In addition to well characterized toxin of *L. monocytogenes*, there are other proteins and surface structures that determine the virulence of *L. monocytogenes*. Polymorphonuclear leucocytes (PMN) are essential for resolution of infections with *L. monocytogenes*. Human neutrophils react to extracellular listerial exotoxins by rapid cell activation (Lammerding *et al.,* 1992). Listeriolysin is centrally involved in triggering degranulation and lipid mediator generation. Other factors affecting the pathogenicity of *L. monocytogenes* are; its capacity for intracellular growth, possession of proteins sequestrating iron from ferritin, the presence of catalase and superoxide dismutase, surface components and hemolysin, indicating that it's virulence is multifactorial. The virulence of the organism may be affected by its growth temperature. Growth of *L. monocytogenes* at a reduced temperature (4˚C) increases its virulence intravenously. The hemolysin of *L. monocytogenes* is

recognized as a major virulence factor and its secretion is essential for promoting the intracellular growth and T-cell recognition of the organism (Farber *et al.,* 1991).

2.5.1 Adhesion factors for infection of *L. monocytogenes*

There are eight virulence factors involved in the adhesion of *L. monocytogenes* to host cells (Camejo *et al*., 2011). Lap which is a surface protein allows for initial adhesion to host cell by interacting with the Hsp60, the host cell receptor. Allows for binding within intestinal host cells, studies done using a mouse model indicate that the lap protein is crucial for full virulence of *L. monocytogenes* (Cahoon and Freitag., 2014). The next contributing gene to adhesion is the *dltA* operon which consists of four genes; *dltA, dltB, dltC* and *dltD*. Mutant mice strains lacking the activation of this operon showed diminished ability to adhere to host cells. This operon is regulated by *VirR*. *FbpA* is exposed at the surface of the host cell. This then binds to immobilized human fibronectin. The expression of *FbpA* affects the proper secretion of LLO and *inlB*, therefore considered a chaperone for the stability of LLO and *inlB* secretion (Camejo *et al*., 2011; Orsi and Wiedmann., 2016; Cahoon and Freitag., 2014).

The *inlJ* gene is only found in the genomes of *L. monocytogenes* and is expressed at the surface of bacteria recovered from liver and blood of infected animals. Adhesion to the epithelial cells is promoted in *L. monocytogenes* and *L. innocua* strains expressing *inlJ (*Camejo *et al*., 2011). *Ctap* is secreted in response to *PrfA* which is the main transcriptional activator of *Listeria spp*. virulence genes. Absence of *Ctap* leads to increased permeability and acid sensitivity of the membrane integrated and host cell adhesion (Cahoon and Freitag., 2014). Another factor responsible for efficient adhesion is *LapB*. *LapB* is absent from those non-pathogenic strains and its expression is regulated by *PrfA*. It is necessary for adhesion and entry into the mammalian epithelial cell lines for intravenous or orally infected mice. *RecA*, regarded as playing an accessory role to cell adhesion, it contributes to the acid and bile resistance of *L. monocytogenes* (Camejo *et al*., 2011). Increased *RecA* transcription is detected following exposure to low pH and increased bile concentrations. It appears to be more involved in bacterial colonization of the human gastro-intestinal tract and translocation of the intestinal epithelium.

The *inlF* gene is a virulence factor specific to *L. monocytogenes*. It promotes increased host cell binding and entry under specific conditions. It is currently undergoing further studies; however, it is

believed to only be active during infection of specific host species. A study conducted by Kirchner and Higgins, 2008 were able to determine the impact of *inlF* in *L. monocytogenes* infection. Using the Drosophila as a test organism, knockout experiments were done using small GTPases Rho1 which is the homolog of RhoA in mammalian cells. RhoA and Rho kinases (ROCK's) have major downstream targets such as serine-theronine protein kinases which are involved and regulated cell functions such as cell adhesion, motility, contractility, gene expression and cytokinesis. It was further found that the knockout of expression and ability of ROCK's allowed for greater infection and virulence of *L. monocytogenes* in mamamalian cells (Kirchner and Higgins, 2008). Host cells are treated with CT04, which is a transferase exoenxyme C3 isolated from *Clostridium botulinum* with Y27632 which works by inactivating ROCK as it is a specific inhibitor. Genetic screening was used to identify *L. monocytogene* mediating factors to ROCK activity which plays a role in infection. A 2.2Kb *L. monocytogenes* DNA insert was found to code *inlF* which belongs to the multi-gene internalin family however its function still remains unknown (Kirchner and Higgins, 2008). Cells, whose ROCK activity was inhibited, showed the expression of *inlF* and resulted in a 3 fold increase in virulence and infection rate. It is therefore believed that *inlF* can increase infection rate and virulence of *L. monocytogenes* when ROCK activity is inhibited.

2.5.2 Invasive factors for infection of *L. monocytogenes*

There are seven invasive factors used for entry of *L. monocytogenes* into the host cell. The first proteins identified as mediators of *Listeria* entry into non-phagocytic cell types is the internalins, *inlA* and *inlB* (Orsi and Wiedmann., 2016). These belong to the internalin family. The host cell receptor for *inlA* is E-cadherin which is a trans-membrane glycoprotein. *inlA/E*- Cadherin interaction is species-specific. Ninety six percent of human clinical strains express full length functional form of *inlA* while only 65%of food-isolated strains contained fully formed length form. The critical role of *inlA/E*-cadherin is epithelial cell invasion (Camejo *et al*., 2011; Orsi and Wiedmann., 2016).

Vip is anchored to the peptidoglycan by *SrtA* and is present in all *L. monocytogenes* lineages, absent from non-pathogenic species. It is needed for the entry into several epithelial cell lines. The endoplasmic reticulum-resident chaperone, Gp96, was identified as the host cell receptor for *Vip*. Also noted is the interaction of Vip-Gp96 may possibly interfere with toll-like receptors (TLR) which then leads to the control of the innate immune response by *L. monocytogenes* (Camejo *et al*.,

2011). Autolysin encoded by the *aut* gene aids entry of *L. monocytogenes* into various mammalian epithelial cells. A mutation in autolysin, rendering it inactive, shows a great decrease of viable bacteria after intravenous infection of mice and oral inoculation of guinea pigs (Cahoon and Freitag., 2014). It is the virulence factor that is believed to be responsible for the maintenance and control of *L. monocytogenes* surface architecture when being exposed to host cells and composition of products released from the bacterial surface (Cahoon and Freitag., 2014).

A surface protein called p60 is encoded by the *iap* gene (Camejo *et al*., 2011). This protein has murine hydrolase activity and without it, cells become deformed and hooked shaped. A study showed that introducing p60 in mutant *iap* cells, restoration of cell internalization occurred. *Igt* is diacyglyceryl transferase responsible for lipidation of prolipoprotiens which contribute to faster cell multiplication and also contribute to the net charge of the bacterial surface (Orsi and Wiedmann., 2016). LpeA is a 35kDa homolog of *S. pneumoniae* PsaA, a lipoprotein used in streptococcal cell adherence. LpeA aids *L. monocytogenes* entry into murine hepatocytes and human intestine epithelial cells (Camejo *et al*., 2011; Orsi and Wiedmann., 2016; Cahoon and Freitag., 2014).

Listeriolysin-O (LLO) plays an important role in bacterial vacuole evasion but also induces the influx of calcium ions for the invasion of epithelial cells (Orsi and Wiedmann., 2016). This influx of calcium ions have also shown to cause a transient mitochondrial network fragmentation which slows down the host cells bio-energetic state and thus allowing a most transient entry of *L. monocytogenes* into the host cell.

2.5.3 Virulence factors involved in the escape from the host vacuole

Listeriolysin-O (LLO) is a protein belonging to the cholesterol-dependent cytolysin (CDC) toxin family. The gene encoding LLO is the *hly* which is found on a locus containing the main virulence factors (*prfA, plcA, hly, mpl, actA* and *plcB*) (Orsi and Wiedmann., 2016). Activity of *L. monocytogenes* is best at acidic pH, however, movement towards neutral pH this prevents excessive damage to the host. The interaction between LLO binding to the host cell membrane, as well as, the facilitation of *Listeria* entry via cystic fibrosis transmembrane conductance regulator (CFTR), suggests that *Listeria* exploits mechanisms of cellular ion homeostasis to escape the phagosome (Orsi and Wiedmann., 2016). In Figure 3, the point of activation for the various genes involved in

infection with *L. monocytogenes* can be seen from entry into the host cell, multiplication within the cell as well as finally expulsion from the host cell.

Besides the characterised Listeriolysin-O (LLO) encoded by the *hly gene, L. monocytogenes* also produces two other hemolysins; phosphatidylinositol-specific phospholipase C (Pl-PLC) encoded by the *plcA* gene and phosphatidylcholine-specific phospholipase C (PC-PLC) encoded by the *plcB* gene (McLauchlin *et al*., 2004). Unlike the LLO which lyses host cells by pore formation, these virulence factors act by disrupting the phagosomal membrane lipids. The bacterium also produces zinc (2+) dependent protease, which acts like an exotoxin. Mutation in the encoding gene (*mpl*) reduces virulence in mice (Camejo *et al*., 2011).

PrsA2 which is a post-translocation chaperone aids in the folding of proteins translocated across the bacterial membrane. Absence of PrsA2 shows instability of LLO and PC-PLC. An accessory function of PrsA2 is efficient *Listeria* intracellular replication. SvpA is a 64kDa protein that is covalently bonded to the cell wall by sortase B (SrtB). SvpA mutants virulence is strongly impaired in mice and therefore promotes bacterial escape (Camejo *et al*., 2011; Orsi and Wiedmann., 2016; Cahoon and Freitag., 2014). Finally, a signal peptidase, *Lsp*, is responsible for the maturation of *L. monocytogenes* lipoproteins. Any mutation to the *Lsp* gene results in the decreased ability of *L. monocytogenes* to escape from the phagosomes. ActA has also been implicated in escape from the vacuole however the exact mechanism by which this occurs is not fully understood.

2.5.4 Intracellular survival and multiplication of *L. monocytogenes*

L. monocytogenes has the remarkable ability to replicate as if growing in pure culture once it has escaped the vacuole. In order to meet its energy requirements, *L. monocytogenes* relies on glucose-1-phosphate within the host cyctoplasm. This process on dependent on a hexose phosphate transporter protein, Hpt. Hpt allows for replication and it is vital for proliferation in mouse organs. Hpt makes a crucial modification to the E2 subunit of pyruvate dehydrogenase in the presence of limiting concentrations of available host lipoyl substrates (Cahoon and Freitag., 2014). A gene encoding a pyruvate carboxylase, pycA, is used in the tricarboxylic acid cycle. Any mutation to this gene results in the inability to replicate within mammalian macrophages and epithelial cells. Another important factor for virulence and optimal bacterial growth during infection is Fri, which is uniquely involved in iron storage (Cahoon and Freitag., 2014). A mechanism used by vertebrates is

to ensure that all bacterial pathogens encounter iron deprivation upon entry into the cell. RelA is shown to play a critical role for bacterial intracellular growth in non-phagocytic cells and macrophages. Mutant *relA* in *L. monocytogenes* results in an inability to accumulate (p)ppGpp in response to amino acid starvation. Proteomic analyses unveiled that Prsa2 is necessary for optimal viability of *L. monocytogenes* within host cell cytosol. Also PrsA2 shows adaptive survival under certain conditions (Cahoon and Freitag., 2014).

Recently discovered in *Listeria* was the role of small non-coding RNA (sRNA) and other regulatory RNA needed for virulence and growth within macrophages (Cahoon and Freitag., 2014). A total of 29 sRNA and anti-sense RNA are expressed intracellularly and studies on mutants of these sRNA show that these mutants are attenuated in mice models. Studies therefore revealed the expression of sRNA is vital for growth of the bacteria in macrophages (Cahoon and Freitag., 2014).

2.5.5 Cell-to-cell spread and intracellular motility

ActA is the major virulence factor of *L. monocytogenes* as it is responsible for the polymerization of actin filaments at one pole of the bacterial cell. This actin filament resembles a comet tail allowing for propulsion within the host cells cytosol (Orsi and Wiedmann., 2016). Cell-to-cell spread is brought upon by this movement. ActA alone is sufficient for intracellular motility even in absence of other *Listeria* factors. The stimulation of actin polymerization is achieved by ActA mimicking the WASP family proteins which are the host cells actin nucleating factors (Cahoon and Freitag., 2014). Interaction between ActA and VASP (Vasodilator-stimulated phosphoprotein) play a key role in infection. VASP is an actin cytoskeletal regulatory protein and in mice models, mutants were ActA and VASP are unable to interact show avirulence and the inability to be motile within the host cell cytosol (Camejo *et al*., 2011; Orsi and Wiedmann., 2016; Cahoon and Freitag., 2014).

2.5.6 inlA/B operon

An operon called *lmBA* encodes a 20kDa protein located on the bacterial surface. The protein, LMaA induces delayed type hypersensitivity and other CMI responses. There are six *Listeria monocytogenes* virulent genes, namely; *prfA, pclA, hlyA, mpl, actA*, and *plcB* located together in one virulence gene cluster between the house keeping gene idh and *prs* (Seveau, 2014).

2.5.7 ActA

ActA (the *actA* gene product) is a surface protein required for intercellular movement and cell to cell spread through bacterially induced actin polymerization. Aided by the listerial membrane surface protein ActA, from the gene, *actA*, invasion and mobilization of the intracellular bacterium occurs when actin assembly is initiated inside a host cell (Seveau, 2014). The bacterial membrane-bound ActA protein then recruits the Arp2/3 complex. Once recruited, Arp2/3 helps to polymerize actin filaments at the posterior end of the bacterium, creating an actin comet tail within the host cell and propelling the bacterium forward. This function allows the bacterium to move throughout the host cell cytoplasm as well as to invade neighboring eukaryotic cells (Camejo *et al*., 2011).

CK-0944636, a molecular inhibitor can be used to bind Arp2/3 within its hydrophobic core and preventing its active conformation (Camejo *et al*., 2011). This compound can be used to inhibit formation of actin filament comet tail by *L. monocytogenes* and is used as a way to study Arp2/3 complex in living cells.

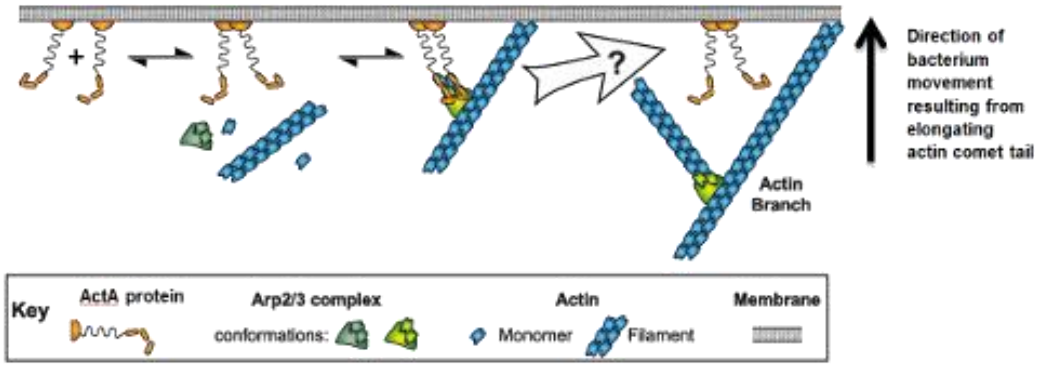

Figure 2: ActA protein, tethered to the bacterial membrane, stimulates actin filament nucleation with the Arp2/3 complex. This generates branched arrays of filament that grow towards the membrane to which they are tethered, elongating the actin strand and moving the bacterium, adapted from Camejo *et al*., 2011.

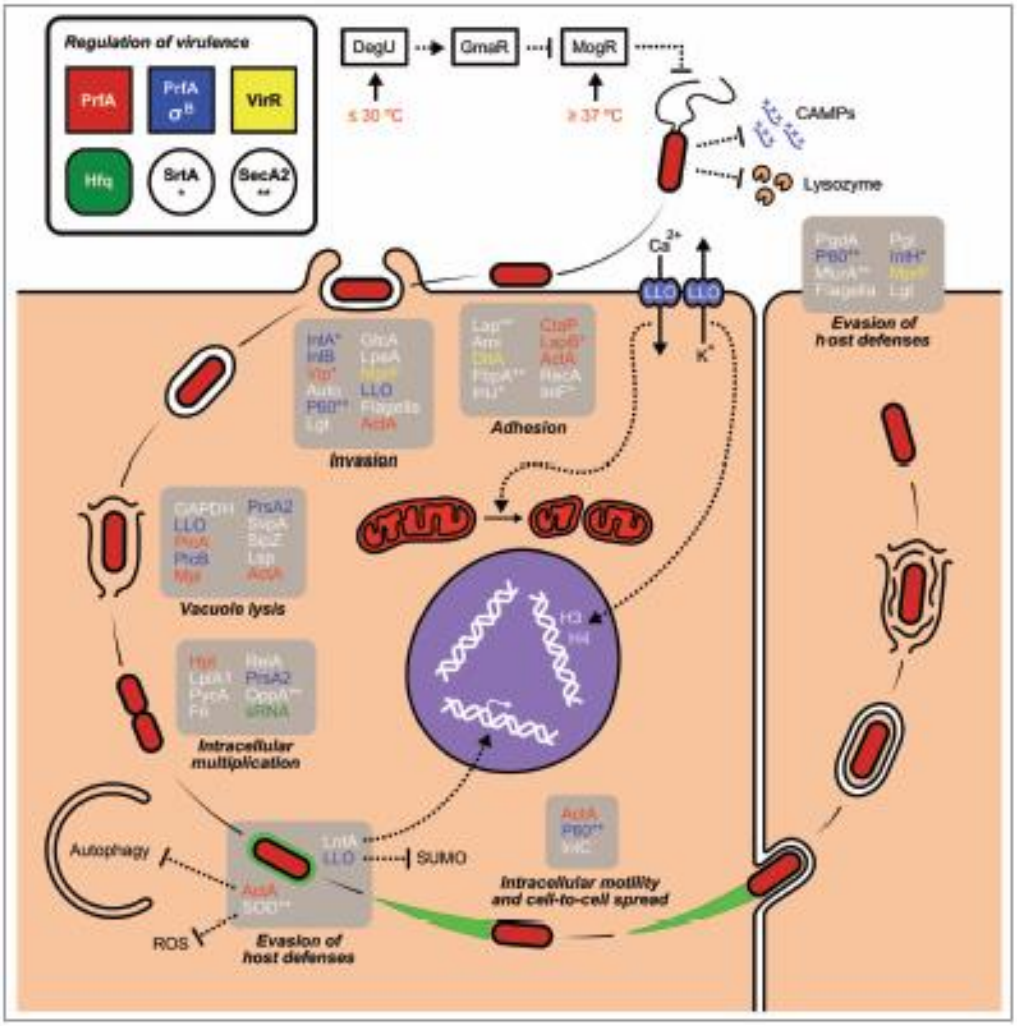

Figure 3: Infectious cycle of *Listeria monocytogenes* as well as the virulence factors involved, adapted from Camejo *et al*., 2011.

2.6 Epidemiology

2.6.1 Epidemiology

A variety of food products have been involved in most outbreaks, including soft cheese (Bille *et al.*,1990) and cooked meat products (Aguado *et al.*, 2004). These are considered to add a special risk due to the ability of *Listeria* to grow and survive in them. However, there are other products, traditionally considered of low risk, which have recently been linked to listeriosis transmission, such as the large listeriosis outbreak reported in Italy due to the consumption of corn. Though no fatalities occurred, more than 1500 people were affected (Aguado *et al.*, 2004).

The incidence of listeriosis appears to be on the increase worldwide, with a significant number of cases, especially in Europe. The annual endemic disease rate varied from 2 to 15 cases per million populations, with published rates varying from 1.6 to a high rate of 14.7 in France for 1986. Zambia had 85 reported cases of meningitis due to *Listeria* (Aguado *et al.*, 2004). In Togo, 8 out of 342 healthy slaughter animals were positive for *L. monocytogenes* (serovars 1/2a and 4b) isolated from the intestinal lymph nodes (Hohne *et al.*, 1975). In Northern Nigeria, 27% mortality rate due to *L. monocytogenes* (serovar; 4) was reported (Onyemelukwe *et al.*, 1983). In Bangui (Central African Republic), a study was conducted on primary and opportunistic pathogens associated with meningitis in adults, in relation to human immunodeficiency virus serostatus. In this study, 276 HIV-positive patients enrolled and 215 patients had cryptococcal meningitis and the bacteria and fungi involved in meningitis did not display high levels of in vitro resistance. Conventional microbiology techniques failed to detect the causative agent of meningitis cases. A broad range bacterial PCR detected DNA from *Streptococcus pneumoniae* in three samples, *Neisseria meningitides* in two, *Escherichia coli* in one, *Listeria monocytogenes* in two and *Staphylococcus aureus* in one (Bekondi *et al.*, 2006).

The number of human carriers of *L. monocytogenes* as assessed by the examination of faecal samples ranges from as low as 0.5 % to as high as 69.2 % or 91.7 % (11 of 12 laboratory technicians). At any one time, around 5 to 10% of the general population could be carriers of the organism. The use of the newer methods, however, may show the carrier rate to be significantly higher. On the other hand, it was found that pregnant women with stools positive for *L. monocytogenes* never delivered an infant with listeriosis. Thus, because of the high rate of clinically healthy carriers, the presence of *L. monocytogenes* in feces may not necessarily be an indication of infection (Farber *et al.*, 1991).

In a study of the duration of faecal excretion, it was found that of 12 people examined over 16 months, 11 excreted *L. monocytogenes* on one or more occasions: one for 6 months , one for 4 months , three for 3 months , four for 2 months and two for 1 month . However, no one excreted the same serotype of *L. monocytogenes* in the feces for a consecutive period of longer than 2 months. It is apparent that although shedding patterns tends to be erratic among different individuals, carriers in some cases can shed the organism for long periods. Although among animals the carrier rate is generally considered to be 1% to 5% (range 1% to 29%), recent studies involving newer methods

for isolating *Listeria* species have indicated that much higher carriage rates may also occur (Farber *et al.,* 1991).

A study done by Pérez- Trallelo, 2014, looked at the number of listeriosis cases reported in Spain spanning from 200 to 2014. In summary, from January 2009 to December 2012, there were 7-12 annually reported cases of listeriosis. This number increased from January 2013 to December 2014 with a total of 27 reported cases. Oddly most of the serovars responsible for these outbreaks belonged to 1/2b and not to the more commonly associated 1/2a and 4b serovars. From the 27 infected individuals, 26 suffered from sepsis and 1 with diarrheal disease. Eleven of the 27 infected were pregnant women, whom 8 of their children were also infected. From the 8 children, 5 new-borns became ill, 2 were miscarriages and 1 was still born. Since 2013, 35 reported cases originated from Gipuzkoa, Northern Spain. A total of 6 deaths were reported.

As reported by Hernandez-Milian and Payeras-Cifre, 2014, Table 1 counts the percentage of mortality due to *L. monocytogenes* in USA, China, Denmark and Spain from as early as 1964 to 2012. These figures give an indication that *L. monocytogenes* is still present globally and is still a major concern as the mortality rate is relatively high in comparison to the number of individuals infected.

Table 1: Mortality due to *L. monocytogenes* over four countries adapted from Hernandez-Milian and Payeras-Cifre, 2014.

Countries	**Years**	**Death (%)**
USA	2009-2011	17.6
China	1964-2010	26
Denmark	1994-2003	21
Spain	2011	20-30
Majorca	2002-2012	25
Madrid	1986-2007	24.3
Barcelona	2011	14

2.6.2 *Listeria* in Foods

L. monocytogenes can be found on poultry carcasses and in poultry processing plants. The occurrence of pathogens in chickens in many countries is well documented but their presence on South African poultry products has not been extensively investigated. Two studies investigating contamination of food available from street vendors in Johannesburg have been reported, but in these only 6 of the samples tested were raw poultry (Mosupye and Von Holly., 2000). *Listeria* organisms are documented to be zoonotic; one of the sources of infection is the domestic fowl where it could occur as unapparent infection. The carriage of *Listeria monocytogenes* and other *Listeria* in indigenous birds has not been documented in Kenya (Njagi *et al.*, 2004).

The occurrence of *Listeria* species in various foods, fish and water was investigated and it was found that *L. innocua* was the most common followed by *L. monocytogenes* and the water was contaminated by feces. Listeriosis may be caused by all 9 serovars of *L. monocytogenes*, however most cases are due to serovars 1/2a, 1/2b and 4b. The major 13 food-borne outbreaks of listeriosis, as well as the majority of sporadic cases, have been caused by serovar 4b strains. This suggests that serovar 4b may possess unique virulence properties (Buchrieser *et al.,* 2007). However, geographical differences in the global distribution of serotypes apparently exist. *L. monocytogenes* appears to be a normal resident of the intestinal tract in humans, indicating why antibodies to *Listeria* species are commonly found in healthy people (Charpentier *et al.*, 1999).

Lambetz *et al*., 2010, monitored the occurrence of *L. monocytogenes* in RTE in Sweden. They had reported that an increased trend of *L. monocytogenes* in food products within Sweden encouraged the analysis of RTE meats, fish and cheese in order gain deeper knowledge into the then existing occurrence of the organism. A total of 1590 samples were used to cover the three categories tested and results showed that occurrence of *L. monocytogenes* in RTE meats were at 1.2%, cheese at 0.4% and fish at 12%. A comparison to another Swedish study had reported 64% occurrence of *L. monocytogenes* in meat processing plants which is indicative that control and sanitation of Swedish food processing plants needs to be revised in order to curb the high percentages of *L. monocytogenes*.

2.6.3 *Listeria* in Poultry

Over the past 15 years, persistence of *L. monocytogenes* in food processing plants has been reported from a wide range of studies (Ferreira *et al*., 2014). The persistence of this organism in other food associated processes results in transmission the of *L. monocytogenes* from these food processing plants onto food resulting in contamination as well as transmission to humans as a pathogen (Ferreira *et al*., 2014). Researchers have also been able to isolate specific strains that adapt in order to remain in an environment such as the development of biofilms etc. (Ferreira *et al*., 2014). Due to these mechanisms and adaptations, over the years many countries have reported listeriosis outbreaks in humans such as the United States, Japan, United Kingdom, Chile, Canada, Sweden, Australia and Germany (Ferreira *et al*., 2014). With the consumption of poultry as an easily available source of meat and protein, the three most occurring pathogens to contaminate poultry are *Salmonella, Campylobacter* and *Listeria* species (Brizio and Prentice, 2015). Although it is rare for poultry flocks to vector listeriosis outbreaks, the occurrence of *L. monocytogenes* in poultry broiler farms are relatively high (Dahshan *et al*., 2016). The study done by Dashan et al., 2016 in Egypt reported isolation of *Listeria* species from poultry farms at 47.5% where *L. monocytogenes* accounted for 1%. A study conducted by Bouayad *et al*., 2015 in Algeria reported *L. monocytogenes* occurrence at 8.9% across three broiler abattoirs sampled. These studies amongst others (Brooks *et al*., 2016 and Elmali *et al*., 2016) have been able to create a link between broiler poultry and the contamination of *L. monocytogenes*.

In 1996, Ojeniyi *et al*., conducted a study on the isolation of *L. monocytogenes* from 3080 samples spread across seven abattoirs. Isolation rate varied from 0.3% to 18.7% from the different abattoirs. When considering caecal samples from broiler flocks, occurrence was 4.7%. The total caecal sample pool was 2078. It was then concluded that *L. monocytogenes* are primarily localized at broiler production abattoirs.

Bouyard *et al*., 2015 conducted a study at two different stages of a broiler abattoir for the detection of *L. monocytogenes*. The two points of sampling were after evisceration and after the final product has been refrigerated at 4°C for 24 hours. From a total of 212 samples, *Listeria* occurrence was recorded at 46.7% and *L. monocytogenes* was recorded at 8.9%.

A study done in Brazil, Rio Grande de Sul by Mendonca *et al*., 2016 reported occurrence of *L. monocytogenes* in frozen chicken meat at 33.3%. Serovar occurrence was at 66% for the ½ serotype with half of the isolates being 1/2a and the other half 1/2b.

Kosek-Paszkowska *et al*., 2005, in Poland, conducted a study to compare occurrence of *L. monocytogenes* obtained from chicken parts to its occurrence obtained from minced meat. In chicken parts, 51.7% of the samples were contaminated with the organism and 30.4% contamination in minced meat. A total of 14% of the samples were *L. monocytogenes* contaminated.

2.7 Antimicrobials

2.7.1 Discovery of Antimicrobials

Antimicrobial drugs have greatly enhanced human life expectancy, reduced mortality and improved quality of life and almost won the war against many infectious diseases. An antimicrobial is a substance that is able to inhibit or destroy microorganisms, with the largest group being those that are effective against bacteria (Prescott *et al.,* 2000). It was the discovery by Fleming in 1929 of the antibiotic penicillin, a fungal metabolite, and its later development by Ernst Chain and Howard Florey during World War II that lead to the antibiotic revolution with the subsequent discovery and development of many other classes of antibiotics. Antibiotics are the "miracle drugs" that are extensively used for the treatment and prevention of infectious diseases in humans and pets, as well as in food-producing livestock, poultry and fish. Today, antibiotics play a major role in modern agriculture and livestock industries and their use has been on the rise in many developing nations (Sarmah *et al.,* 2006). The Centers for Disease Control and Prevention (CDC) estimates that approximately 22 700 Kg of antibiotics are produced in the United States alone each year, with roughly 40% used in agriculture. Europe gradually started decreasing the use of antibiotics in food producing animals, especially performance enhancers, Sweden and Denmark banned avoparcin in 1986, followed by the European Union (EU), in 1995. In 1999, the growth-promoting use of bacitracin, spiramycin, tylosin and virginiamycin were banned in the EU (Phillips, 1999).

Previously, in developing countries antimicrobial drugs were used as performance enhancers on a limited scale, nowadays, many developing countries such as India, China and South Africa use huge quantities of antibiotics as growth promoters. Many antimicrobial drug classes are used in animals for prophylaxis and therapy. This use tends to increase where farm management is not optimum or when endemic diseases are not properly controlled. Several guidelines are available for appropriate use of antimicrobial drugs in animals, but very little is being done in developing countries (Byarugaba, 2004).

2.7.2 Antimicrobials used in the global poultry industry

Antibiotics have been widely used in the poultry industry since their discovery more than 50 years ago. They represent an extremely important tool in the efficient production of animal products such as meat and eggs (Phillips *et al.*, 2004). They are used by the poultry industry and poultry veterinarians to enhance growth and feed efficiency and reduce bacterial diseases (Donoghue, 2003). Antimicrobial classes used as therapeutics in the poultry industry include: aminoglycosides, tetracyclines, β-lactams, fluoroquinolones, macrolides, polypeptides, amphenicols, sulphonamides and trimethoprim (Stolker and Brinkman, 2005).

2.7.2.1 ß-Lactams (cephalosporins and penicillins)

The β-lactams have been categorized into five classes as described by Bush (1989). Penicillins were first discovered by Alexander Flemming in 1928 with the drug being available on the market in 1946. Penicillins are either natural or synthetic being derived from fungi and work by binding to enzymes to cell wall cross linking proteins, thus preventing cell wall synthesis. Currently the penicillins are grouped into 5 orders; (1) natural penicillin, (2) penicillinase-resistant penicillins, (3) amniopenicillins, (4) extended spectrum penicillin and (5) aminopenicillin/beta lactamase inhibitor combinations (Silvers and Spires, 2002). Penicillin G (natural penicillin) is an effective antimicrobial for Gram-positive bacterial infections in poultry. The Gram-negative bacteria causing respiratory tract infections in birds, namely *Pasteurella multocida, Avibacterium paragallinarum*, *Escherichia coli* and *Gallibacterium anatis* (previously called *Pasteurella anatis* causing septicaemic lesions in chickens) can also be treated with ampicillin and amoxicillin. However, penetration of the respiratory tract with this hydrophilic antibiotic may be poor. Penicillin G is available both orally and parental form, having a short half-life of 20-30 minutes, in cases of treatment it is often used for continuously administration. The broad-spectrum β-lactams such as amoxicillin are more effective for Gram-negative infections such as *E. coli* airsacculitis. Ceftiofur is the only cephalosporin approved for use in poultry in the United States (Silvers and Spires, 2002). It is commonly administered with Marek's disease vaccine to day-old chicks (Kinney and Robles, 1994).

Ampicillin which falls under the aminopenicillin family is capable of penetrating both Gram-positive and Gram-negative bacteria. Essentially it is able to inhibit the inhibitor enzyme transpeptidase which in returns affects the final stage of cell wall synthesis in binary fission (Miller,

2002). This leads to cell lysis and is why at times ampicillin is referred to as being bacteriolytic. Amoxicillin is also in the family of aminopenicillins and is similar to ampicillin in that both antibiotics interfere in cell wall synthesis. Amoxcillin targets penicillin binding proteins (PBP's) which are enzymes responsible for the cross-linking bacterial cell walls (Miller, 2002). PBP's are found anchored to the cell membrane to which the β-lactam ring binds to the BPB's. This results in the inability to synthesize cell walls and again ultimately leads to cell death. Cefotaxime belonging to the cephalosporin group of β-lactams also acts in inhibition of cell wall synthesis. One advantage of cefotaxime over penicillin and amoxicillin is that it is resistant to beta-lactamses due to its structural configuration. This implication leads to its use over a broad range of beta-lactamase producing bacterium (Miller, 2002).

2.7.2.2 Aminoglycosides and Aminocyclitols

Three aminoglycosides are used in poultry: gentamicin, neomycin and streptomycin. Neomycin is commonly used to treat enteric infections and is administered either in feed or water (Farouk *et al*., 2015; Tulkens *et al*., 1999). Gentamicin is the most widely used aminoglycoside and it is used subcutaneously in day-old chicken or turkey chicks. Streptomycin is partially absorbed from the intestine and therefore can be used to treat systematic *E. coli* infections. Spectinomycin and hygromycin are the two poultry approved aminocyclitols. Spectinomycin is highly effective for *E. coli* infections when combined with lincomycin (Smith *et al.*, 2007). Aminoglycosides are used for the treatment of serious infections commonly caused by gram-negative bacilli however when used in combination with other agents, it is affective against gram-positive infections. The basic mechanism of action for aminoglycosides is the binding to the aminoacyl site of 16S ribosomal RNA which is found within the 30S ribosomal subunit. This leads to inhibition of translocation by misreading of the genetic code. Elongation fails to occur due to the interruption in the mechanisms involved in ensuring transcription accuracy (Farouk *et al*., 2015; Tulkens *et al*., 1999).

2.7.2.3 Quinolones and Fluoroquinolones

Quinolones are an important group of synthetic antibiotics with bactericidal action that results from the selective inhibition of bacterial DNA synthesis. They are used in poultry against many Gram-negative bacteria (Stolker and Brinkman, 2005). Quinolones were first used to treat urinary tract infections however now it is the most commonly prescribed antibiotic in the world. Quinolones can treat both Gram-positive and gram-negative bacterial infections (Aldred *et al*., 2014). The targets of

quinolones are bacterial type II topoisomerases, gyrase and topoisomerase IV. The founding member of the quinolones drug class is nalidixic acid which was first isolated by George Lesher in 1962. The second generation of quinolones compounds was developed in 1980's highlighted by norfloxacin, ciprofloxacin and ofloxacin which displayed a considerably improved activity against gyrase and greater penetration into gram-positive organsims (Aldred *et al*., 2014).

The fluoroquinolones are second generation quinolones that are highly effective against Gram-positive, Gram-negative and *Mycoplasma* infections. This was brought about by the introduction of a fluorine at position C6 and a major ring substitution (piperazine or methyl-piperazine) at C7. This is why quinolones are often referred to as "fluoroquinolones". Enrofloxacin is a fluoroquinolone with a good respiratory tract distribution can eliminate *Mycoplasma gallisepticum* infection in laying hens. Its use is banned in the USA as it readily induces resistance to it in the zoonotic *Campylobacter spp* (Aldred *et al*., 2014). Gyrase and topoisomerase IV modulate the topological state of DNA. Both gyrase and topoisomerase IV generate staggered cuts in the DNA backbone. They produce double-stranded breaks in the bacterial chromosome. Quinolones takes advantage of this by increasing the concentration of enzyme-DNA cleavage complexes, turning gyrase and topoisomerase IV into cellular toxins (Aldred *et al*., 2014). Resistance to quinolones are classified into target-mediated resistance, plasmid-mediated resistance and chromosomal-mediated resistance. Target-mediated resistance is associated with mutations to gyrase and topoisomerase IV. Plasmid-mediated resistance, are plasmids which have found to carry resistance genes to quinolones as well as resistance genes to other antibiotics. These plasmids commonly confer ≤10 fold resistance however there have been cases reported in which resistance to quinolones due to plasmids are as high as ≥250 fold. Chromosomal-mediated resistance is caused by mutations to regulatory proteins involved drug uptake via protein channels called porins. When the expression of porins is down-regulated by the cell, low-level resistance to quinolones is seen (Aldred *et al*., 2014).

2.7.2.4 Tetracyclines

The tetracyclines are the most widely used antimicrobials in poultry and was first discovered in the 1940s. This is largely due to their affordability, a wide margin of safety and broad-spectrum (*Mycoplasma*, Gram-positive and Gram-negative bacteria) and intracellular activity (Barile *et al*., 2012). They are easily administered *en mass* in either feed or water and have limited side effects. The three tetracyclines most commonly used in poultry are chlortetracycline, oxytetracycline and

doxycycline (Smith *et al.*, 2007). The basic mechanism of tetracyclines as an antibiotic is its ability to prevent the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site, thus preventing protein synthesis. Majority of the vast *tet* genes are involved in interactions with the efflux, a smaller amount involved with ribosomal protection and finally *tet*(X) interacting with enzymatic processing (Chopra and Roberts., 2001). There is however a few *tet* genes whose exact function and mechanism in antibiotic resistance remains unknown. Due to the vastness of tetracycline genes, genes have been ordered to have the same gene designation if their amino acid sequence identity is ≥80% (Novais *et al*., 2012). Two genes are considered different if they have ≤79% amino acid sequence identity. The ribosomal protection proteins confer greater resistance to tetracyclines as compared to the efflux *tet* genes (Barile *et al*., 2012; Chopra and Roberts., 2001; Novais *et al*., 2012).

2.7.2.5 Glycopeptides

This class of antibiotics is used for the treatment against severe infections by Gram-positive bacteria. Currently there are undergoing new developments in this drug class due to the rapid increase in vancomycin-resistant enterococci (VRE) and vancomycin-resistant *S. aureus* (VRSA) in the 20th century. Structurally, glycopeptides are constituted of a glycosylated cyclin/ polycyclic non-ribosomal peptide which is produced by many groups of filamentous actinomycetes (Harakeh *et al*., 2009).

The basic mechanism of action of glycopeptides is to interfere or prevent efficient cell wall synthesis and thus results in the cell dying. Structural support of the cell is given by peptidoglycan monomers, these antibiotics work by passing through the cell membrane, moving to the site of polymerization (Harakeh *et al*., 2009). At this site, they form non-covalent bonds with terminal carbohydrates which results in the inhibition of cross-linking by the trans-peptidase. The bacterial cell wall now weakens as it is unable to hold up positive osmotic pressure within the cell, resulting in cytolysis. Vancomycin, the antibiotic used in this study, acts by targeting the D-Ala-D-Ala terminus of pentapeptide precursors (Lungu *et al*., 2011). This forms a complex with the D-Ala-D-Ala residues by creating 5 hydrogen bonds with the peptide backbone of the glycopeptide. Due to the steric hindrance caused by this complex, transpeptidation reactions are prevented (Lungu *et al*., 2011).

2.7.2.6 Sulphonamides and trimethoprim

Sulphonamides are bacteriostatics that are used as veterinary drugs for prophylactic and therapeutic purposes; they also act as growth-promoting substances and are commonly administrated in drinking water as coccidiostats. Trimethoprim is a potentiator when administered together with sulphonamides as both act on different enzymes in the folic acid metabolic pathway (Balizs, *et al.*, 2003).

2.7.2.7 Amphenicols

Chloramphenicol (CAP) is active against a variety of pathogens. Although CAP was, previously, widely used in veterinary and human medicine, reports of aplastic anemia in humans arising from its use led to its ban for use in food-producing animals throughout most of the world. Thiamphenicol and Florfenicol, which have structures similar to CAP were permitted as substitutes, the latter is used to treat *E. coli* airsacculitis infections in poultry (Corcia, *et al.,* 2002). The mechanism of action for amphenicols is the inhibition of the 50S ribosomal subunit in bacterial cells. The ribosome itself is comprised of two subunits, the 50S and 30S (Corcia, *et al.*, 2002).

Drugs which act upon the inhibition of protein synthesis are broad and are classified depending on which subunit is targeted, that is, either the 50S or 30S ribosomal subunit (Aase *et al*., 2000). 50S inhibitors work by physically blocking initiation of protein translation or translocation of peptidyle-tRNA's. Chloramphenicol acts by preventing protein synthesis without affecting any other major metabolic processes (Aase *et al*., 2000). This depends on the configuration and conformation of the molecule, with special priority given to the propanol moiety of the compound. Any structural changes made to the propanol moiety of the compounds drastically reducing its antibiotic capabilities however changes to other parts of the molecules show very small effects (Aase *et al*., 2000).

2.7.2.8 Macrolides

Macrolides are drugs with a macrocyclic lactone ring of 12 or more elements. These include antibiotics, antifungal drugs, prokinetics and immunesuppressants (Brenciani *et al*., 2009). Macrolides have excellent tissue penetration and antimicrobial activity against gram-positive pathogens. Erythromycin was the first macrolide introduced from Streptomyces. Erythromycin is

most frequently used in poultry to treat *Staphyloccus aureus* arthritis. Tylosin and Tiamulin are considered to be highly effective in the treatment of *Mycoplasma* infections in laying hens to restore egg production and reduce transovarian transmission (Brenciani *et al*., 2009). The only poultry approved lincosamide is lincomycin, it is primarily used to treat infectious coryza and infectious synovitis. It is commonly used to treat *Clostridium perfringens* induced necrotic enteritis and also to enhance poultry performance (Smith *et al*., 2007). Macrolides work in the following ways; inhibition of adherence, inhibition of virulence factors, inhibition of biofilms and inhibition of quorum sensing (Brenciani *et al*., 2009).

2.7.3 The use of antimicrobials as performance enhancers

The earliest evidence of the growth promoting effects of antibiotics became apparent in chickens exposed to small doses of chlortetracycline which grew more rapidly than non-exposed chickens (Stockstad, 1950). The growth enhancing effect of this broad- spectrum antibiotic class seems to be more marked (Jukes and Williams, 1953) than those, e.g. bacitracin and virginiamycin, with a primarily Gram-positive spectrum (Jukes, 1955). However, tetracyclines are considered to have a negative impact on the commensal microflora of the intestine and therefore their use as performance enhancers is not recommended. In poultry, performance enhancers, such as bacitracin and virginiamycin can also control *Clostridium perfringens* infections, which are potentially fatal. Estimates suggest that the average benefit of such products is an improvement in feed conversion rate (FCR) of approximately 3%, with a range of 0-5 % (Bedford, 2000). The mechanism of action of antibiotics as growth promoters is related to interactions between the antibiotic and the gut microbiota, thus the direct effects of antibiotic growth promoters on the microflora can be used to explain decreased competition for nutrients and reduction in microbial metabolites that depress growth (Dibner and Richards, 2005).

Before the middle of 1980s in Europe, antibiotics which were authorized to be included in poultry feeds without a veterinary prescription were tetracyclines, avoparcin, flavophospholipol, avilamycin, bacitracin methylene dislicylate, zinc bacitacin, lincomycin, spiramycin and virginiamycin (Castanon, 2007). Because of the risk concerning residues of antibiotics in edible tissues and products that can produce allergic or toxic reactions to consumers and the potential risk for humans, the WHO (1997) and the Economic and Social Committee of the European Union (1998) concluded that the use of antimicrobials in food animals is a public health issue (Castanon,

2007). This led to the total banning of antimicrobials, with the exception of sulphonamides, as performance enhancers in poultry by the EU in 2006 (Anadon, 2006).

2.7.4 Rules and regulations of antimicrobial use in poultry production

Governments in many countries have established new institutions, standards, and methods for regulating food safety and have increased investments in hazard control. The policies of antimicrobial residues control in developing countries are mainly aimed at addressing food safety issues (Woodward, 1996). The regulation of the use of antimicrobials in food animals vary from country to country. For example the European Union (EU) has strictly regulated control of the use of veterinary drugs, including performance enhancing agents in food-animal species by issuing several Regulations and Directives. The primary consumer safety consideration is addressed via Maximum Residue Limits (MRL), established by Council Regulation EEC/2377/90. The MRL defines the maximum level of residues of any component of a veterinary medicine that may be present in foodstuffs of animal origin without presenting any harm to the consumer (European Commission, 2001).

The EU definition is virtually the same as that adopted by the Codex Alimentarius Committee for Residues of Veterinary Drugs in Foods and the approach to evaluation of residues of veterinary medicinal products within the European Union is very similar to that employed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) that evaluates for Codex Alimentarius. The MRL for any substance is determined from data submitted by manufacturers or suppliers to the Safety of Residues Working Party, a sub-committee of the Committee for Veterinary Medicinal Products (CVMP). This determination is ratified by the CVMP and adopted into law by a Regulatory Committee in the form of Commission Regulations (Woodward, 1996; European Commission, 2001). For veterinary medicines used in food animal species in the EU the MRL is determined by an iterative process from a range of safety data, the most important of which is the Acceptable Daily Intake (ADI). The ADI is defined as the level of a substance that may be consumed daily without presenting a hazard to the consumer. It is based on a suitable no observed effect level (NOEL) or from observations in humans, divided by a safety factor, often 100 (Woodward, 1996; European Commission, 2001). Even if efforts have been made to harmonize the MRL at world level [under the aegis of World Trade Organization (WTO)] and the Codex

Alimentarius), it must be acknowledged that the latter still strongly differs from one geographical area to another (Prescott, 1997). Thus, due to these MRL differences, the same chlortetracycline-based medicine is granted, for a given species, a withdrawal time of 7 days in Canada and zero in the USA. Agricultural use of antimicrobials in the USA and Canada is also regulated. There are three categories of use: as feed antimicrobials, as over-the counter drugs and as veterinary prescription drugs. Feed antimicrobials include performance enhancers, coccidiostats and therapeutic antimicrobials and are licensed for specific purposes in the case of broilers, young pigs or calves or feedlot cattle (Prescott, 1997).

In the United Kingdom (UK) and other European Union (EU) countries, antimicrobials are authorized as either veterinary medicinal products or zoo technical feed additives. Veterinary medicinal products and performance enhancers are subject to assessment for safety, emergence of antimicrobial resistance, cross-resistance to therapeutic antimicrobials and transferable resistance (Rutter, 1997). Regulating the use of these veterinary drugs in the UK is primarily the responsibility of the Veterinary Medicines Directorate (VMD) (Al-Ghamdi *et al.*, 2000).

China has regulated the use of antimicrobials in animal feed since 1989 and only non-medical antibiotics are permitted as feed additives. Antimicrobials used include monensin, salinomycin, destomycin, bacitracin, colistin, kitasamycin, enramycin, and virginiamycin (Jin, 1997). In Russia: bacitracin, grizin, flavomycin, and virginiamycin are registered for use as performance enhancers (Panin *et al*., 1997). Most African countries recognize the importance of food hygiene particularly with regards to meat safety, and have laws and regulations that govern food production and processing including such aspects as meat inspection and drug residue levels.

However, enforcement of such laws and regulations is usually poorly done. Farmers, for instance, can buy veterinary drugs and administer them without a prescription. Firstly, financial resources are usually inadequate for law enforcement agencies to carry out their work effectively. Secondly, support facilities such as laboratories are usually ill-equipped in both equipment and personnel. This is further compounded by the fact that in terms of prioritization for resource allocation, livestock production compared to public health issues is a low priority for most African governments (FAO/WHO Regional Conference, October 2005).

Although, the use of veterinary drugs in The Sudan is regulated by the Pharmacist and Toxics Act, approved in 2001, there are no specific regulations for antimicrobial usage in food producing animals. The act mentioned above deals with the licenses of drug sale and the authority of veterinarian on veterinary drugs (FAO/WHO Regional Conference, October 2005).

2.7.5 The Role of Antimicrobial Residues and Antibiotic Resistance in Food Safety

Residues in food of animal origin result from the feeding or application of antimicrobials, other therapeutic agents, pesticides and heavy metals in livestock (Oehme, 1973). In 1983, a group of internationally renowned experts convened jointly by the Food and Agriculture Organization of the United Nations (FAO) and WHO concluded that "illness due to contaminated food was perhaps the most widespread health problem in the contemporary world," and "an important cause of reduced economic productivity". In 1992, the U.N. Conference on Environment and Development recognized that food was a major vehicle for the transmission of environmental contaminants, both chemical and biological, to human populations throughout the world, and urged countries to take measures to prevent or minimize these threats. In 2000, the World Health Assembly, the supreme governing body of the WHO, unanimously adopted a resolution recognizing food safety as an essential public health function (Unnevehr, 2003).

Residues may have a direct toxic effect on consumers, *e.g*., allergic reactions in hypersensitive individuals (Dayan, 1993; Ormerod *et al.*, 1987; Woodward, 1991), or they may cause problems indirectly through induction of resistant strains of bacteria (Stolker and Brinkman, 2005). In humans, the triggering of allergic reactions in sensitized individuals by penicillin residues is well documented (Dewdney *et al.*, 1991). A rare fatal blood dyscrazia in sensitized individuals can also be triggered by chloramphenicol residues in food (Settepani, 1984).

Since the human outbreak of the zoonotic, multi-antimicrobial resistant *Salmonella* Typhimurium DT104 in 1986, the use of antibiotics in food-producing animals has become a public health issue. The concerns are that not only could humans become infected with difficult to treat bacteria, but that commensal enteric bacteria such as *Enterococcus faecium* can transfer resistance to the intestinal bacteria of humans. Thus concerns about use of antibiotics in animals and their possible

impact on human health cover two major issues: the antibiotic agent that are used and the way in which they are used.

The knowledge on the occurrence, fate and dissemination of antimicrobial residues and antibiotic resistant bacteria is increasing. However, a significant gap still exists in our understanding of the relationship between antibiotic residues, their metabolites and antibiotic resistant bacterial populations after their excretion. To avoid possible extinction, the bacteria have adapted their own defenses against antimicrobials (Levy, 1992). The populations of bacteria with this ability tend to be enhanced when antimicrobials are used to treat disease and can lead to certain infections becoming untreatable *e.g.* multi-resistant *Mycobacterium tuberculosis* infections (Davies, 1997).

Antimicrobials can also have a marked effect on commensal microflora resulting in either an increase in the antimicrobial resistance of these bacteria or replacement of the bacterial populations by more resistant bacteria (Levy, 1992). Of particular interest in the latter is the effect that the ingestion of food of animal origin may have on the intestinal microflora of humans, either via colonization with multi-resistant bacteria or the effect of antimicrobial residues (WHO, 2000). The worldwide increase in antimicrobial resistant bacteria (Morris and Masterton, 2002) has led to social and scientific concern that the over-prescription and misuse of human prescribed antibiotics and the increased and widespread use of sub-therapeutic doses of antibiotics in agriculture are responsible for this trend (Smith *et al.*, 2002).

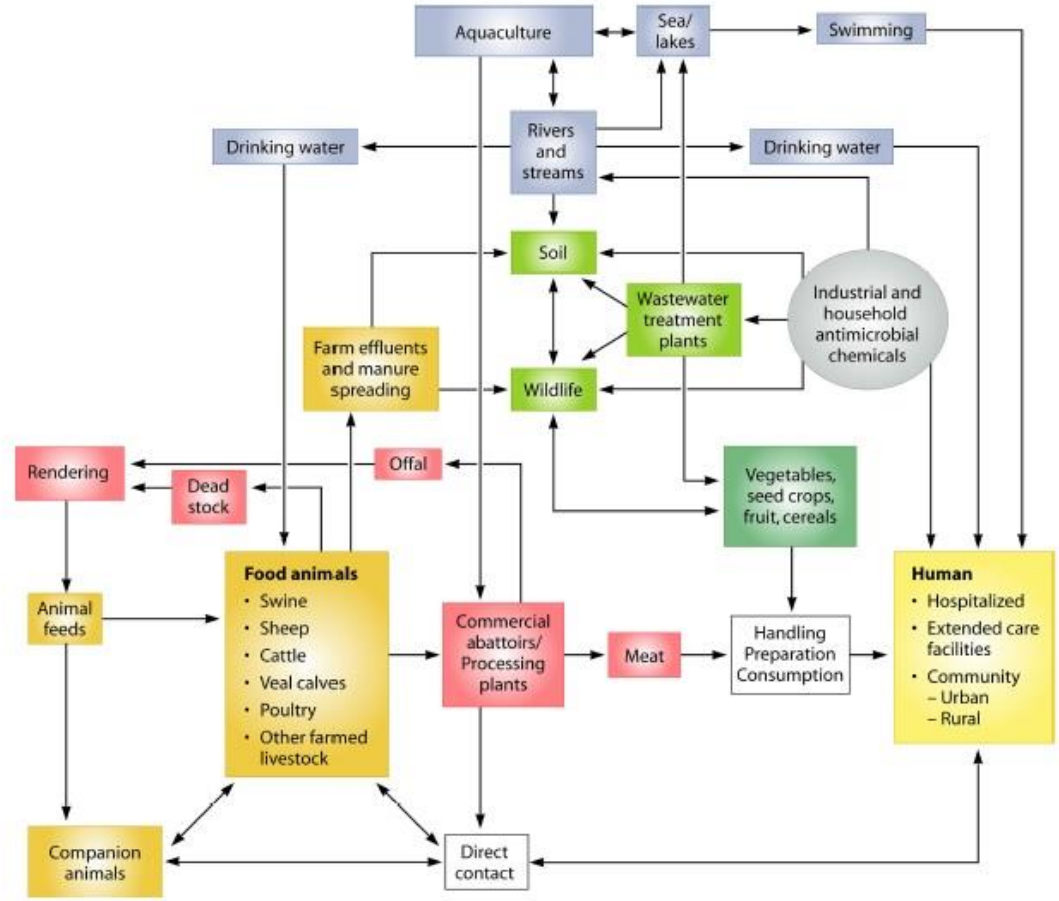

Figure 4: Dissemination of antibiotics and antimicrobials via the environment, waste water treatments, communities, farms and hospitals that results in compounding resistance in *L. monocytogenes* (Adapted from Davies and Davies, 2010).

2.7.6 Antimicrobial resistance in *Listeria monocytogenes*

L. monocytogenes, as well as other *Listeria* spp., are usually susceptible to a wide range of antibiotics (Hof *et al*., 1997). However, evolution of bacteria towards resistance has been considerably accelerated by the selective pressure exerted by over-prescription of drugs in clinical settings and their heavy use as promoters for growth in farm animals (Charpentier *et al.*,1995). Isolation of the first multi-resistant strain of *L. monocytogenes* was in France in 1988 (Poyart-Salmeron *et al*., 1990) and since then multi-resistant *L. monocytogenes* strains have been recovered from food, the environment and sporadic cases of human listeriosis (Hadorn *et al*., 1993;

Charpentier *et al.,* 1995). Antibiotics to which some *L. monocytogenes* strains are resistant include tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim, and rifampicin (Charpentier and Courvalin, 1999).

Tetracycline resistance has been the most frequently observed among *L. monocytogenes* isolates (Charpentier *et al*., 1995; Charpentier and Courvalin, 1999).

2.7.7 The Tetracycline Genes

Six classes of tetracycline-resistance genes; *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(P), and *tet*(S) have been described in Gram positive bacteria (Charpentier *et al*., 1995). However, only *tet*(L) and *tet*(S) have been identified in *L. monocytogenes* (Poyart-Salmeron *et al*., 1992; Charpentier and Courvalin, 1999). *tet*(M) and *tet*(S) confer resistance by ribosomal protection, whereas the *tet*(L) gene codes for a protein which promotes active efflux of tetracycline from the bacteria.

The tetracycline genes *tet*(K) and *tet*(L) are grouped together as they share 59% amino acid sequence similarity. They are primarily found in Gram positive bacteria and these genes encode a protein which confers resistance to tetracycline and chlortetracycline (Chopra and Roberts., 2001). An indication of their presence is gram positive bacteria being resistance to tetracycline but not to minocyclines or glycylcyclines. These genes are usually found on small transmissible plasmids and have been reported to become integrated into the staphylococci or *Bacillus subtilis* genome. It is more common to find small plasmids in staphylococci carrying the *tet*(K) gene rather than in larger plasmids (Novais *et al*., 2012). The *tet*(K) gene is most commonly found in *S. aureus* however it is present in other Staphylococcus species. The *tet*(L) gene have been found in small plasmids related to food borne diseases and share anywhere between 98-99% similarity in their amino acid sequences (Barile *et al*., 2012).

tet(M) and *tet*(O) produce proteins which are responsible for ribosomal protection. Presence of these genes is able to confer a wider spectrum of resistance to tetracyclines as compared to the efflux pump associated genes. The ribosomal protection proteins have homology to elongation factors EF-Tu and EF-G (Chopra and Roberts., 2001). The greatest homology is seen at the N-terminal area, which contains the GTP-binding domain. Both the *tet*(M) and *tet*(O) proteins are able to reduce the susceptibility of ribosomes to tetracycline action. They have ribosome-dependent GTPase activity. *tet*(M) acts by competing with elongation factor G (EF-G) to bind to the ribosome.

The *tet*(O) protein acts by binding GTP and GDP and therefore preventing the action of tetracyclines (Novais *et al*., 2012).

tet(P) gene is unusual because it consists of the *tet*A(P) gene, which encodes a functional efflux protein, linked to the *tet*B(P) gene, which appears to encode a ribosomal protection protein. *tet*A(P) has been found without *tet*B(P), but *tet*B(P) has not been found alone (Novais *et al*., 2012).

tet(S) also falls under the ribosomal protection group of tetracyclines and its mechanism of action is believed to be similar to that of *tet*(M) and *tet*(O). Experiments have been done in order to uncover if *tet*(S) and *tet*(O) is regulated however these studies remain inconclusive (Chopra and Roberts, 2001). Conjugative plasmids or chromosomes are the common source housing the *tet*(S) gene.
Transfer of resistance between *L. monocytogenes* can occur in the gastrointestinal tract of domestic animals where both species live and where sub-inhibitory levels of tetracycline may be expected. In fact, tetracyclines are the second most commonly used antibiotics worldwide. They are used extensively in animal foodstuffs, especially for poultry, and it is noteworthy that tetracycline resistance was the single most common resistance marker in food-borne *L. monocytogenes* isolated from chicken and turkey (Chopra and Roberts, 2001). Antibiotic resistance in *L. monocytogenes* is reaching an era where virtually all antibiotics will be rendered ineffective because of various mechanisms employed by *L. monocytogenes* to counteract the effects of therapeutic agents. *L. monocytogenes* is currently known to form biofilms in utensils and equipment or food processing machinery (Czajka *et al*., 1993).

2.7.8 Antibiotic susceptibility testing

Several types of antimicrobial susceptibility testing methods have been devised. The test used most frequently is the disk diffusion procedure (Kirby-Bauer test), in which clinical interpretations are derived from correlations with the reference test. Mueller-Hinton agar is generally the medium of choice for these tests (Abuin *et al.,* 1994). In the Kirby-Bauer test, the microorganism is spread on the agar surface in order to get a lawn of growth. As soon as the antibiotic–impregnated disk comes into contact with the moist agar surface, water is absorbed into the filter paper and the antibiotic diffuses into the surrounding medium. The zone size that is observed in disk diffusion has no meaning on itself, but standards are used to derive a correlation between the zone sizes and minimum inhibitory concentrations (MICs) of the test organism (Abuin *et al.,* 1994).

Epsilometer test (E–test) consists of antibiotic-impregnated strips that are placed on the surface of the agar. The antibiotic content of the strip is graded and the concentration is printed linearly along the strip. After incubation MIC is read from the point on the strip where the zone of inhibition passes. In contrast to the disk diffusion test, where the orientation of the disk does not matter, placing the E- test strip upside down on the agar will alter the results (Abuin *et al.*, 1994).

Nucleic acid based assay may offer advantages over phenotypic assays. The development of new molecular techniques such as PCR using molecular beacons and DNA chips expand the possibilities for monitoring resistance. Although molecular techniques for the detection of antimicrobial resistance are clearly winning in routine diagnostics, phenotypic assays are still the method of choice for most resistance determinants (Chen *et al.*, 2006).

2.8 Typing of *Listeria*

Phage typing has proven to be a valuable epidemiological tool in investigations of many infectious diseases. Since the initial discovery of phages specific for *Listeria* species in 1945, several groups have assessed the usefulness of phage typing *L. monocytogenes*. Recently a new set of phages derived from both environmental sources and lysogenic strains have been found.

In isozyme typing, bacteria are differentiated by the variation in the electrophoretic mobility of any of a large number of metabolic enzymes. This technique is useful in either confirming or eliminating a common source as the cause of an outbreak of food-borne listeriosis (Farber *et al.*, 1991).

DNA fingerprinting using restriction enzyme analysis (REA) has recently been used to characterize strains of *L. monocytogenes* causing outbreaks of listeriosis associated with Mexican–style soft cheese in Los Angeles, as well as the Nova Scotia and Switzerland outbreaks (Aguado *et al.*, 2004).

Plasmid typing was recently used in conjunction with DNA fingerprint to confirm a case of cross–infection with *L. monocytogenes*. However, this technique is of less importance since *L. monocytogenes* does not appear to carry plasmid. On the other hand *L. innocua* carry plasmids ranging in size from 3 to 55 MDa (Aguado *et al.*, 2004). Monocine typing has recently been evaluated as a typing tool for *L. monocytogenes*. Although this technique is potentially promising as an epidemiological tool, only 59 and 56 % of serovars 1/2a and 4b were found to be producers of monocines. In one instance a pair of *L. monocytogenes* strains isolated from a mother and a

newborn, which could not be phage typed, proved identical by monocine typing (Baloga *et al.*, 1991).

2.8.1 Isolation and Identification of *Listeria monocytogenes*

Significant efforts have been dedicated to the development of enrichment media and protocols for *L. monocytogenes* isolation. Ideal enrichment media would facilitate recovery of injured *Listeria* cells and enrichment of *Listeria* species (*L. monocytogenes*) over competing microflora. In traditional culture–based assays, it becomes very difficult to detect *L. monocytogenes* at any level when it is greatly outnumbered by other *Listeria* species, such as *L. innocua*, which in most cases are present together with *L. monocytogenes* (Bille *et al.,* 1992). Species–specific identification with biochemical standard methods, which include sugar fermentation or the CAMP test, is laborious and time consuming and can require 1 to 2 weeks for identification (Seragusa *et al*., 1990). Moreover, differentiation between species and strains is not always reached (Aguado *et al.,* 2003).

Currently, newer methods (molecular), such as pulsed field gel electrophoresis that has been used to differentiate *L. monocytogenes* from *L. inocua* (Howard *et al*., 1992), random amplified polymorphic DNA (RAPD), real time PCR (Wiedmann *et al.,* 1993) and restriction endonuclease analysis (REA), have been employed to directly characterize the microorganism without the need for isolation (Gudmundsdottir *et al.,* 2005).

2.8.2 Diagnosis

The rapid identification of *L. monocytogenes* is important so that the appropriate antibiotic therapy can be initiated. A diagnostic scheme for the identification of *L. monocytogenes* food borne cells, emerge in 40 hours at 30°C. These are typically large colonies, representative of which are used to advantage as heavy inocula on agar plates for the rapid determination of hemolytic activity and acidification of rhamnose and xylose. Additional tests consisting of phase–contrast microscopy or cell morphology and motility, the catalase production test and the KOH viscosity test in place of Gram staining completes the rapid identification of *L. monocytogenes* (Lachica *et al.,* 1990).

2.9 Detection of *Listeria* exotoxin (Listeriolysin O)

The presence of the listeriolysin gene is restricted to the species *L. monocytogenes*. Listeriolysin therefore appears to play a vital role in enabling hemolytic *L. monocytogenes* to survive and multiply within the susceptible host (Leimester- wachter *et al.,* 1989). DNA hybridization studies

have shown that listeriolysin genes are found in *Listeria* species, such as *L. monocytogenes, L. ivanovii*, and *L. seeligeri*. Immuno-blotting performed with affinity–purified antibody to literiolysin allowed the detection of this protein in supernatants of all three species. In this immunological assay two recombinants, (pLM47 and pLM48) were found to produce a polypeptide of 60KDa which cross-reacted with the anti-sera to produce a hemolytic phenotype on blood agar plates (Leimeister-Wachter *et al.,* 1992). Southern hybridization studies confirmed that gene fragments distinguishing an epidemic associated strain from virulent prototype strain of *L. monocytogenes* belong to a distinct function subset of genes and partially cross-hybridize with other *Listeria* species (Herd and Kocks, 2001).

In the analysis of genomic DNA of *Listeria* by southern hybridization with *hlyA* probes, all strains were isolated and digested with the restriction endonuclease *HindIII*. The 0.8-kb *BamHI* probe that was made up entirely of sequences upstream of the listeriolysin gene was found to hybridize to *L. monocytogenes* strains irrespective of serotype, as well as to the *L. seeligeri* and *L. ivanovii* strains (Sibelius *et al.*, 1999). Other methods that can be employed to detect listerolysin are; hemolysin assays and polyacrylamide gel electrophoresis, imuno-magnetic beads for *Listeria* and *Listeria* exotoxin detection kits (Sibelius *et al.,* 1999).

2.9.1 Treatment

When infection occurs during pregnancy, antibiotics given promptly to the pregnant women can often prevent infection of the fetus or new born. In general, isolates of *L. monocytogenes*, as well as strains of other *Listeria* species, are susceptible to a wide range of antibiotics except tetracycline, erythromycin, streptomycin, cephalosporins, and fosfomycin (Charpentier *et al.,* 1999).

The treatment of choice for listeriosis remains the administration of ampicillin, penicillin G combined with an aminoglycoside. The association of trimethoprim with sulphonamide, such as, sulfamethaxazole in co–trimoxazole, is a second choice therapy (Charpentier *et al.,* 1999). The most active agent in the combination is trimethoprim, which is synergized by sulfamethaxazole. Most isolates from clinical as well foodborne and environmental sources are susceptible to the antibiotics active against gram positive bacteria (Abuin *et al.,* 1994).

2.9.2 Control and Prevention

The risk of acquiring food borne listeriosis has made food producers and distributors to adhere to strict hygienic control measures to minimize contamination by *L. monocytogenes*. Although preventive measures in USA have been effective in reducing cases of listeriosis the production of food free of *L. monocytogenes* is still unrealistic in some foods. This difficulty stems from the ubiquitous nature of this organism and the possibility of cross–contamination between one or several products during processing and the use of antibiotics in animal foodstuffs as growth promoters by some commercial farmers, which consequently result in listerial antibiotic resistance (Abuin *et al.*, 1994). The other difficulty in eliminating *Listeria* is its ability to colonize surfaces by forming biofilms that remain attached to equipment surfaces (Wong, 1998).

2.9.3 Conclusion

In conclusion, it can be seen that although *L. monocytogenes* importance as a food borne pathogen has been known for some time (Osaili *et al*. 2011) this organism continues to plague public health globally. The studies reviewed in this section have shown that antibiotic resistance in *L. monocytogenes* is a growing concern. Also the occurrence of *L. monocytogenes* in foods for human consumption is not low enough to be disregarded as an impending threat. Many other countries have documented the occurrence of this organism in great detail (Mosupye and Von Holly., 2000; Aguado *et al.*, 2004; Bekondi *et al.*, 2006) however a major gap exists in South African literature. Another problem area is that it is known that stringent health and safety protocols cannot be adhered to or enforced in developing countries (Woodward, 1996; European Commission, 2001). Considering these two factors, a review in the occurrence of *L. monocytogenes* within Durban, South Africa, will aid in filling the current gap of knowledge around this organism in South Africa. Determining antibiotic profiles for any *L. monocytogenes* found will also give insight into treatment of listeriosis if an outbreak within this country has to occur.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Study Site

Caeca samples were randomly sampled from 148 live broiler chickens within the Durban area, KwaZulu- Natal province, South Africa. Sampling sites were split into three sectors, local markets (LM), broiler farms (F) and retail stores (RS). Local markets are defined as informal trading points scattered throughout Durban, were the broilers are either obtained as they are home-grown or from private small farms. Broiler farms are those commercial farms that mass distribute chicken to various smaller retail stores as well as private sales. Finally, retail stores are defined as franchise stores found with malls and centers that are believed to sell the most hygienic broiler both cooked and frozen, however, sampling of cooked poultry was not considered in this study. For this study, abattoirs were not considered as retail stores obtain their broilers via many different abattoirs and both the local markets as well as broiler farms sell their broilers as live birds and slaughtering of the broilers are done by the consumer. The three locations that were sampled were based on consumer's preference to purchase broiler chickens from these locations. Sterile Swabs were used to collect these caeca samples from live birds which were then placed on ice and transported to the University of KwaZulu- Natal, Westville campus for genetic analyses. A total of approximately 50 random samples were collected from each of the 3 sampling sites. Sampling occurred between March 2015 to May 2016 for the local markets and retail stores. Samples were incubated in 0.1% w/v buffered peptone water for 24 hours at 37°C. Thereafter 5ml of the enriched sample was added to Tryptic Soy Broth (TSB), the second pre-enrichment broth and incubated for 24 hours at 37°C.

Figures 5 and 6 show the locations of the three sources as well as the sub locations for each store (RS) and local market (LM) sampled. The rationale behind sampling these locations are due to these sources being the most common for the purchasing of broiler chickens in Durban.

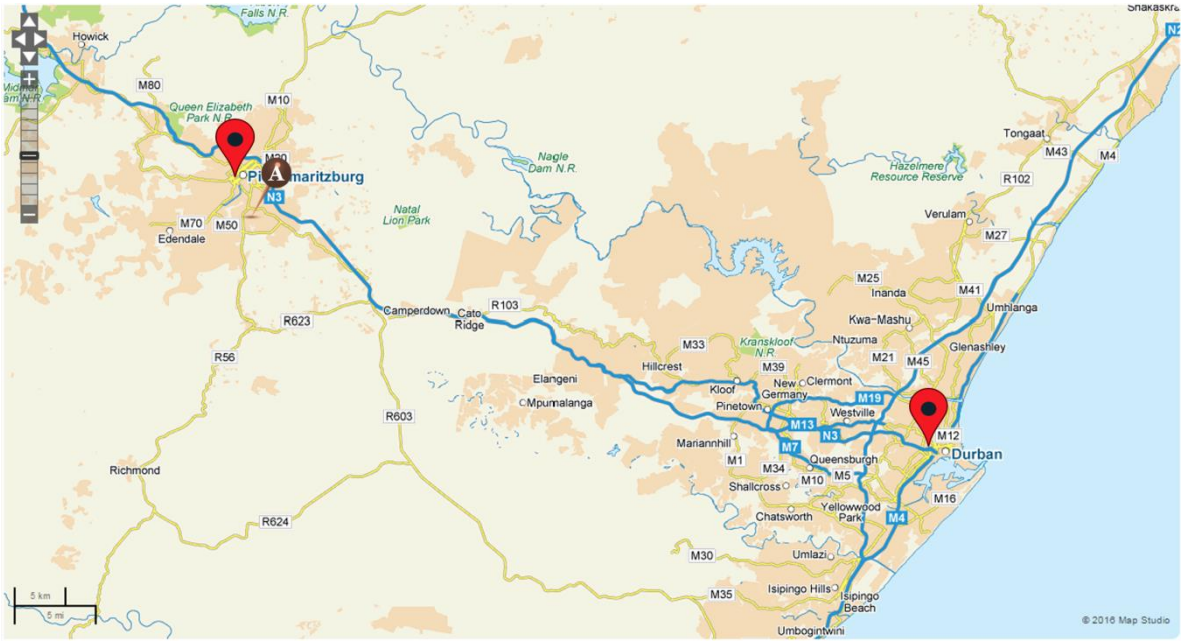

Figure 5: Sample collections points as shown on a map of KwaZulu-Natal. The marker on the left shows the collection point in Pietermaritzburg and the marker on the right indicates the collection point in Durban.

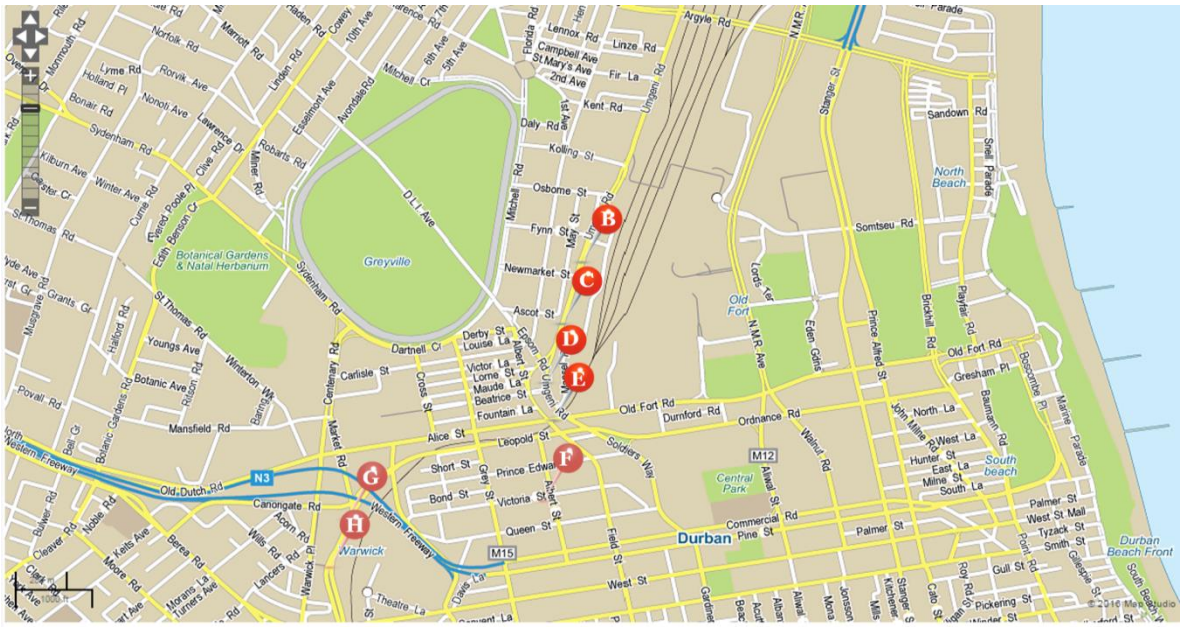

Figure 6: An expanded view of the collection point in Durban, markers areas B, C, D and E are all Retail Stores (RS) and markers F, G and H are the Local Markets (LM) sampled.

3.1.1 Selective enrichment and plating

Following pre-enrichment, selective enrichment was done using the OXOID *Listeria* Selective Broth Base with supplement SRO142E. Five milliliters (5 ml) of the enriched TSB sample was added into 10ml of OXOID *Listeria* Selective Broth and supplement to incubate at 37°C for 48 hours.

Using OXOID *Listeria* Selective Agar Base (LSA) with supplement SRO140E, 0.1µl of the *Listeria* Selective broth was spread plated aseptically onto the agar. These plates were then incubated between 24 and 48 hours depending on the growth observed on the plates after 24 hours. Colonies of interest were those with a black center, surrounded by a grey/ white halo, typical of *L. monocytogenes* (Al-Zeyara *et al*., 2010).

3.1.2 DNA extraction

Total genomic DNA was extracted using the STE buffer method as described by Cheng *et al*. (2003). This entailed the following steps: scraping of a single colony of presumptive *L. monocytogenes* was added to 50µl of STE buffer within an eppendorf microcentrifuge tube. This was then vortexed on high speed for 5 minutes in order to allow disruption of the cell walls. Upon vortexing, 50µl Chloroform was added to the microcentrifuge tube. Centrifugation for 5 minutes at 13 000 xg was carried out and checked for a visible separation of two aqueous layers. The supernatant (DNA and STE buffer) was removed and nandropped using the NanoDrop 2000 to check purity of the DNA. Only samples with a DNA yield equal to or greater than 400ng/ml was used in downstream applications.

3.2 Virulence gene testing

Two virulence genes were used to confirm the presence of *L. monocytogenes*. These were *hlyA* and *inlB*. The *hlyA* gene is responsible for coding the LLO virulence factor and is highly conserved in the two pathogenic strains of *Listeria*. The *inlB* gene which is found on the inlA/B operon was chosen again for its highly conserved sequence as well as it being responsible for coding internalin A and B (Farber *et al*., 1991; Lammerding *et al*., 1992; Sibelius *et al*., 1999). The rationale behind choosing these two genes is that they are responsible for *L. monocytogenes* virulence; mutants of these two genes show the inability to infect cells. The gene primer sequence is listed in the table

below together with the expected product size. Conditions for the *hlyA* amplification were as follows 95°C for 30 seconds, 95°C for 3 minutes, annealing at 58°C for 1 minute and a final extension at 72°C for 3 minutes. Conditions for the *inlB* amplification were as follows; 95°C for 30 seconds, 95°C for 3 minutes, annealing at 57°C for 1 minute and final extension at 72°C for 3 minutes.

Table 2: Primers used in PCR for the testing of virulence genes in isolates

Name of Gene	**Forward and Reverse Sequence**	**Expected amplicon Size (base pairs)**	**Source**
***hlyA*(16sRNAgene)**	**Forward**: AAGCTGTTACTAAAGAGCAGTTGCAAGC **Reverse**: CTGGCAAATAGATGGACGATGTGAAATG	702	Usman *et al*., 2016
inlB	**Forward**: AAAGCACGATTTCATGGGAG **Reverse**: ACATAGCCTTGTTTGGTCGG	146	Kim and Bhunia., 2008

3.3 Antibiotic Resistant Testing

Antibiotic resistant testing and profiles were done in two stages. Firstly, four genes and primer sets were tested using PCR on samples that were positive as *L. monocytogenes* as well as presumptive *Listeria* as seen as colonies on the *Listeria* Selective Agar. Secondly, all positive and presumptive *Listeria* underwent antibiotic resistant profiling using ten antibiotics.

3.3.1 Antibiotic Resistant Genes

Table 3: Primers used for amplification of antibiotic resistant genes from *Listeria* species

Gene	Forward and Reverse Sequence	Expected amplicon size	Source
***tet*(S)**	Forward: ATCAAGATATTAAGGAC Reverse: TTCTCTATGTGGTAATC	156bp	Morvan *et al*., 2010
***tet*(M)**	Forward: GTGGACAAAGGTACAACGAG Reverse: CGGTAAAGTTCGTCACACAC	406bp	Malhotra-Kumar *et al*., 2005
***erm*(TR)**	Forward: GAAGTTTAGCTTTCCTAA Reverse: TTTCCACCATTAACA	190bp	Morvan *et al*., 2010
***msr*(A)**	Forward: GCAAATGGTGTAGGTAAGACAACT Reverse: ATCATGTGATGTAAACAAAAT	401bp	Morvan *et al*., 2010

The *tet*(S) and *tet*(M) genes are responsible for ribosomal protection and are known to confer greater resistance against tetracyclines as opposed to the efflux *tet* gene with the exception of *tet*(B) as explained in the literature review . It is this rationale that was used for selecting these two genes.

The methylase gene, *erm*(TR), is efflux-mediated. It works by target site modification to posttranscriptional methylation of an adenine residue in 23S rRNA which eventually results in co-resistance to macrolides, lincosamides and streptogramin B antibiotics (Varaldo *et al*., 2009). The *msr*(A) gene encodes a protein with a 2 ATP-binding domains characteristic of ABC transporters. The transmembrane component of the MsrA pump remains unknown (Leclerq, 2002).

3.3.2 Antibiotic susceptibility profiling

Ten antibiotics were tested against 27 presumptive *Listeria spp*. samples. The Kirby-Bauer disc diffusion method was used as described by the Food and Drug Administration CLSI M45 document (2015). Antibiotics used are listed in Table 4 alongside the breakpoint values as described by the Food and Drug Administration CLSI M45 document, 2015. The criteria used for the selection of the antibiotics were dependent on three factors: the availability of the antibiotic within the University, the use of these antibiotics to treat *L. monocytogenes* infections and the budget allocated for the purchasing of the antibiotics. The incubation period of the Muller-Hinton agar plates with *L. monocytogenes* were not supplemented with CO_2.

Table 4: The antibiotics used according to the Kirby-Bauer Disc diffusion, as well as, concentrations and breakpoints.

Antibiotic	**Minimum Inhibition Concentration (MIC) ug/ml**	**Zone Diameter Interpretive Criteria (nearest whole mm)**			**MIC Interpretive Criteria (µg/ml)**		
		S	**I**	**R**	**S**	**I**	**R**
Erythromycin	15	≥21	16-20	≤15	≤0.25	0.5	≥1
Penicillin	10	≥17	14-16	≤13	≤8	16	≥32
Vancomycin	30	≥17	15-16	≤14	≤4	8-16	≥32
Cefotaxime	30	≥26	23-25	≤22	≤1	2	≥4
Gentamycin	10	≥15	13-14	≤12	≤4	8	≥16
Rifampicin	5	≥20	17-19	≤16	≤1	2	≥4
Ampicillin	10	≥17	-	≤16	≤8	-	≥16
Kanamycin	30	≥18	14-17	≤13	≤16	32	≥64
Amoxycillin	20	≥20	-	≤19	≤2/1	-	≥4/2

Chloramphenicol	30	≥18	13-17	≤12	≤8	16	≥32

Key: **S**- Susceptible, **I** Intermediate and **R**- Resistant.

The antibiotic discs were aseptically placed onto Muller-Hinton agar spread plated with presumptive *Listeria* grown according to the 0.5 McFarland standards. The 0.5 McFarland standards was provided by the University of KwaZulu- Natal, Microbiology Department and verification of sample to the 0.5 McFarland standard were done by visual means. These plates were then incubated upside down at 37°C for 16 hours before being viewed for zones of inhibition. Five antibiotic discs were placed on each plate. Control used for testing the antibiotics were *E. coli* ATCC 25922 obtained from the Department of Microbiology, University of KwaZulu- Natal.

3.3.3 Statistical Analyses

In order to analyze the data obtained from the Kirby-Bauer Disc Diffusion assay, statistical analysis was carried out using the Statistical package for the Social Science (SPSS) version 16. Firstly, each sample that was tested by the Kirby-Bauer Disc Diffusion Assay was separated according to their respective sources i.e. the Local Markets (LM), Poultry Farms (F) and Retail Stores (RS).

Thereafter two comparisons were made; one which compared the resistance, intermediate and susceptibility profiles of each isolate within one source against the ten antibiotics used and the second being a comparison of resistance, intermediate and susceptibility profiles for the ten antibiotics used against the three different sources. Depending on the zone of inhibition obtained, samples were scored 1 (resistant), 2 (intermediate) and 3 (susceptible) to the antibiotics. Selecting options for cross-tabulation and Chi-Square significance, the software was able to carry out the two comparisons and output p-values that were later used to indicate statistical significance between isolates and the sources. Percentages of resistance, intermediate and susceptibility were also provided which was later used when assessing the profiles of the six classes of antibiotics used.

CHAPTER 4

4 RESULTS

4.1 Polymerase Chain Reaction- *L. monocytogenes* Identification

Figure 7, shows amplification of the positive control for both the *hlyA* and *inlB* gene. *hlyA* produced an amplicon of 700bp whereas *inlB* amplicon is seen at 146bp, both amplified at the expected size.

From a total of 148 samples, 7 showed amplification for the *hlyA* virulence gene as seen in Figure 8. In comparison to the agar plates, 27 showed presumptive *L. monocytogenes* colonies.

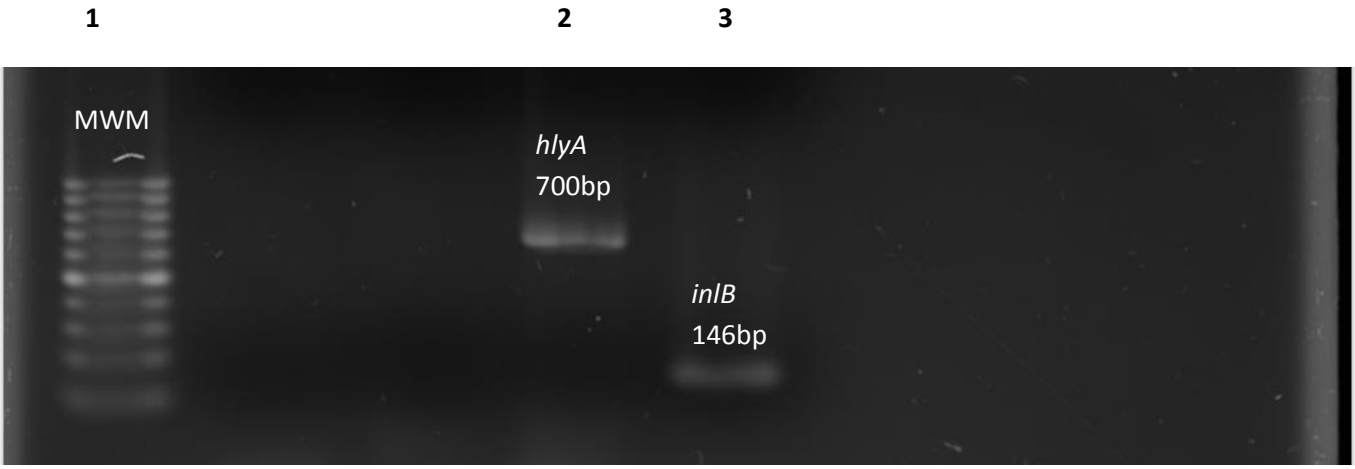

Figure 7: The positive control amplification of the *hlyA* and *inlB* gene on a 1.5% agarose gel stained with ethidium bromide, molecular marker of 100 bp was used.

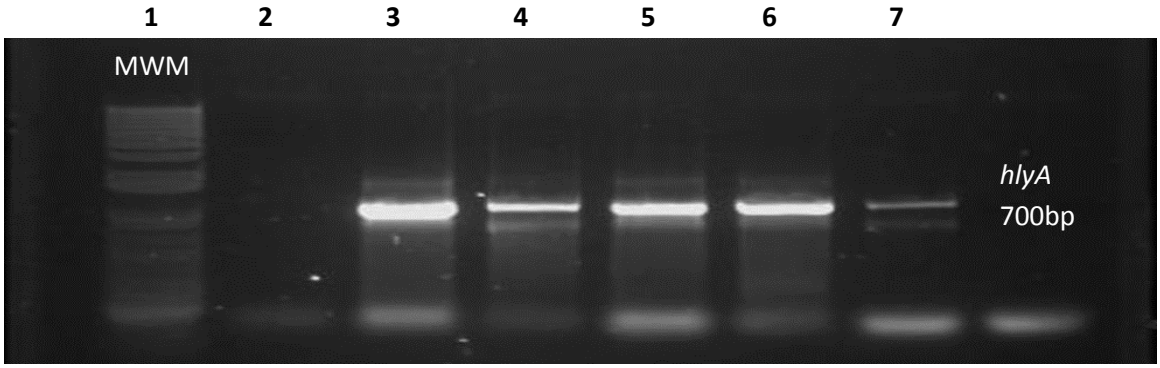

Figure 8: Positive *hlyA* amplication in 7 of the 148 samples run on a 1.5% agarose gel stained with ethidium bromide, molecular marker of 100 bp was used.

Figure 9 shows the positive amplification of the virulence gene *inlB* in 7 samples from a total of 148 tested.

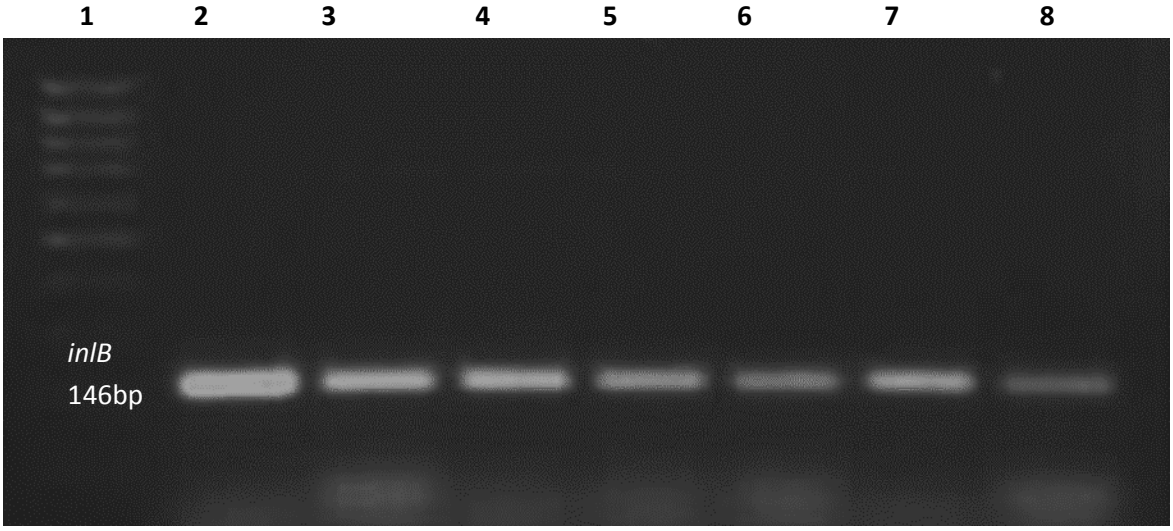

Figure 9: Positive PCR amplication of *inlB* gene from 7 of the 148 samples run on a 1.5% agarose gel stained with ethidium bromide. Molecular weight marker in lane 1 is of 100 bp.

On completion of PCR on the 148 samples, seven were positive for the *hlyA* gene and seven positive for the *inlb*.Interestingly, seven samples showed amplification for the *hlyA* gene as well as the *inlB* gene. However there were no common isolate which showed both the amplification of the *hlyA* gene and the *inlB* gene.

This gives a total of 14 positive *L. monocytogenes* from 148 chicken caeca samples. No sample tested had amplified both the *hlyA* and *inlB* gene which is interesting as it is commonly expected that the presence of the *hlyA* gene would infer the presence of the *inlB* gene (Jacquet *et al*., 2002). This may be due to the presence of the *inlA* gene or other virulence genes which may also confer internalin resistance.

From the total of 27 presumptive *L. monocytogenes* agar plates, 14 were verified by PCR amplification. The remaining 13 plates may have growth of *L. innocua*, as *L. innocua* grows faster than *L. monocytogenes* on *Listeria* Selective Agar (Bruhn *et al*., 2005).

4.2 Polymerase Chain Reaction: Antibiotic Resistance Genes

In this study four antibiotic resistant genes were tested on the 27 presumptive *Listeria* plates. The genes tested against were: *tet(S)*, *tet(M)*, *msr(A)* and *erm(TR)*. Upon numerous amplification trials, only one out of the four antibiotic resistant genes showed amplification i.e. *tet(S)* at 146bp.

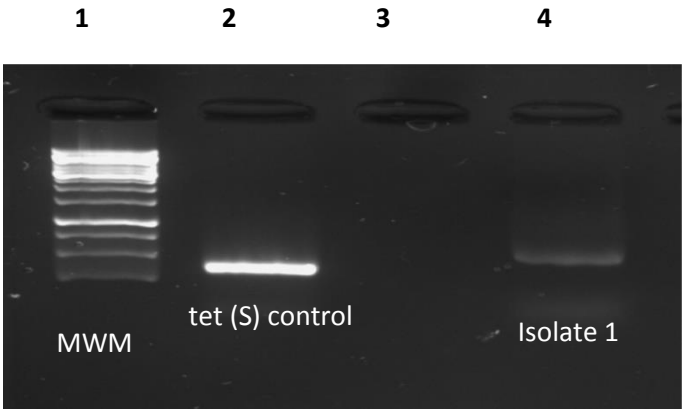

Figure 10: Control amplification of the *tet(S)* gene (lane 2) alongside with amplification from isolate 1 (lane 4), stained with ethidium bromide and run on a 1.5% agarose gel. Molecular weight marker of 100bp was used.

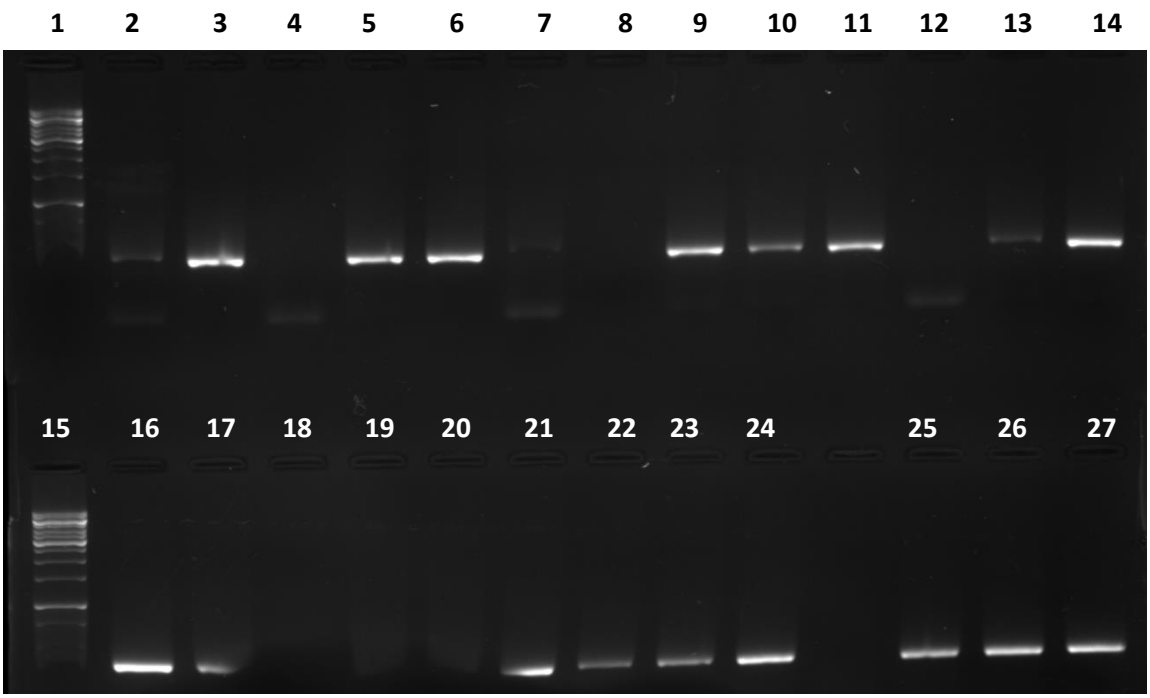

Figure 11: Amplification of the *tet(S)* resistant gene in 18 of the 27 samples tested by PCR, stained with ethidium bromide and run on a 1.5% agarose gel. Lane 1: Molecular Weight Marker (100bp), Lane 2-14: Positive amplicons of the *tet(S).*

In total 19 of the 27 presumptive *Listeria* showed amplification for the *tet (S)* resistant gene. The PCR results can be summarized as seen in Table 6.

Table 5: Occurrence of both presumptive *Listeria* and confirmed *L. monocytogenes* differentiated by source.

Source	**Presumptive *Listeria* species (%)**	**Confirmed *L. monocytogenes* (%)**
Retail Stores (RS)	6 (22)	2 (14.3)
Broiler Farms(F)	8 (30)	9 (64.3)
Local Market(LM)	13 (48)	3 (21.4)

Table 6: A summary of the results obtained from the PCR for the amplification of *hlyA, inlB* and *tet(S).*

Gene	**Positive Isolates**	**Negative Isolates**	**Percentage positive**	**Percentage negative**
hlyA	7	141	5	95
inlB	7	141	5	95
tet(S)	19	8	70	30

4.3 Antibiotic Susceptibility Profiling

Antibiotic susceptibility testing was carried out on the 27 presumptive *Listeria* isolates, against 10 commonly used antibiotics. Figures 12, 13 and 14 show the resistance, intermediate and susceptibility profiles according to the three different sample sites. The highest overall resistance across all sources sampled was seen against ampicillin followed by penicillin and vancomycin with resistant counts at 80.8%, 80% and 73.1% respectively. Although the resistance seems high, none of these were statistically significant (P>0.05). The isolates were most susceptible to gentamicin, kanamycin and erythromycin however, again they showed no statistical significance (P>0.05). Further analysis consisted of multi-drug resistant profiling in which any isolate resistant to more than two classes of antibiotics was considered to be multi-drug resistant.

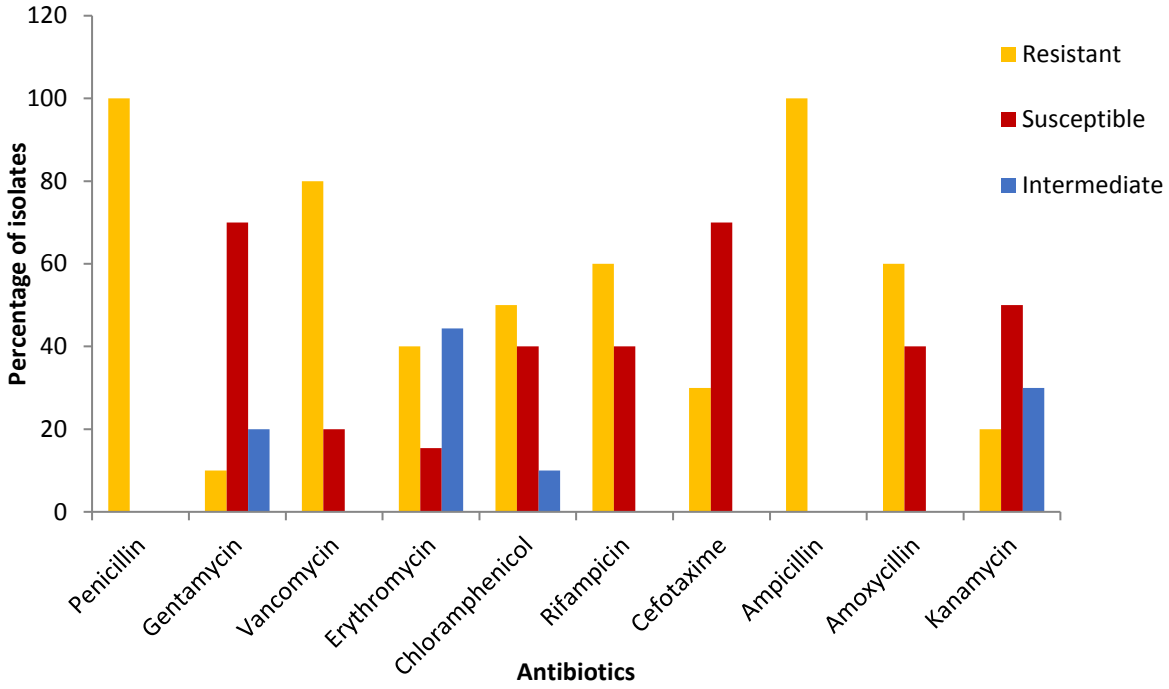

Figure 12: Graph showing the percent of isolates resistant, susceptible and intermediate to the ten antibiotics tested from Local Markets.

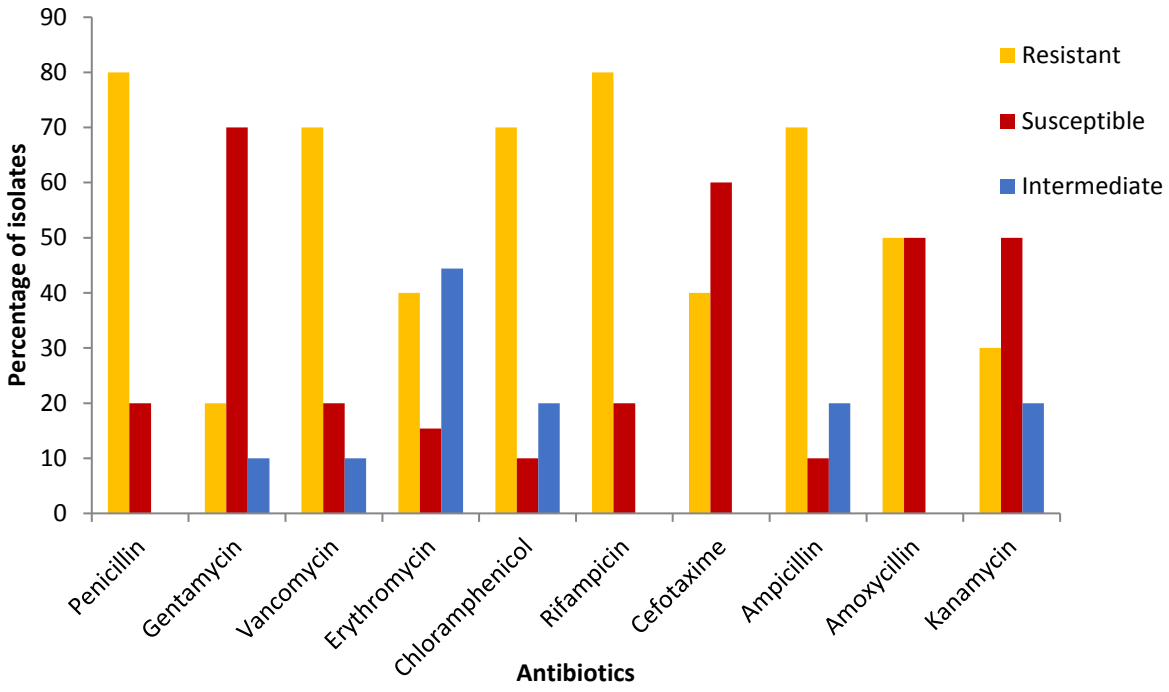

Figure 13: Graph showing the percentage of antibiotics resistant, intermediate and susceptible to the 10 antibiotics tested within Poultry Farms.

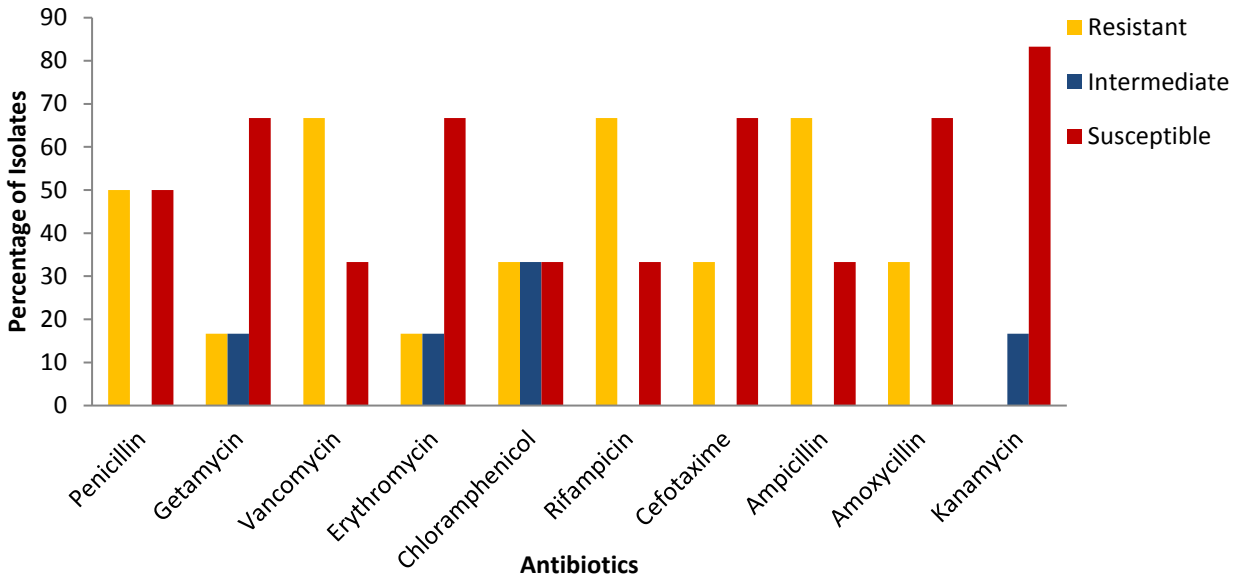

Figure 14: Graph showing the percentage of antibiotics resistant, intermediate and susceptible to the 10 antibiotics tested within Retail Stores.

Table 6 shows each of the 27 isolates and their respective resistant profiles to the 10 antibiotics used. Three categories were designated, those that were confirmed via PCR positive for the *hlyA* gene and thus *L. monocytogenes* isolates. The second category has also been assigned to positive *L. monocytogenes*, due to the positive amplification of the *inlB* gene. The third category is assigned for the presumptive *Listeria* species, although no amplification occurred via PCR, these isolates fit the typical description of *L. monocytogenes* when plated onto LSA.

Table 7: Resistant, intermediate and susceptibility profiles for each of the 27 isolates.

Isolate	Resistant	Intermediate	Susceptible
1	P, V, R, Amp and Amx	Amx	G, E, C, CTX, Amp and K
2	P, C, R, CTX and Amp	Amx and K	G, V and E
3	P, G, V, R and	-	E, C, CTX, Amx and K

Confirmed *Listeria* by PCR (*hlyA*)		Amp		
	4	P, V, E, C, R, CTX, Amp	Amx	G and K
	5	P, E, R, CTX and Amp	V, C and Amx	G and K
	6	P, R, CTX and K	C and Amp	G, V, E and Amx
	7	P, G, V, C, R, CTX	K	E, Amp and Amx
	8	P, V and Amp	G, E, C and Amx	R and CTX
	9	P, V, C, R and Amp	G, Amx and K	E and CTX
Confirmed *Listeria* by PCR (*inlB*)	10	P, V, E and Amp	-	G, C, R, CTX, Amx and K
	11	P, V, C and Amp	-	G, E, R, CTX, Amx and K
	12	V, R, CTX	C and Amx	P, G, E, Amp and K
	13	P, V, E and Amp	C	G, R, CTX, Amx and K
	14	P, V and Amp	G	E, C, R, CTX, Amx and K
	15	P, V, E and Amp	Amx	G, CC, R, CTX and K
	16	P, V, C and Amp	-	G, E, R, CTX, Amx and K

Presumptive *Listeria* from LSA plating	17	P, E, C, R, CTX and Amp	K	G, V and Amx
	18	P, V, E, C, R and K	Amp and Amx	G and CTX
	19	P, G, V, C, CTX, Amp and K	Amx	E and R
	20	P, V, C, R and Amp	Amx	G, E, CTX and K
	21	P, V, R and Amp	E, Amx and K	G,C and CTX
	22	E, C, R and Amp	-	P,G, V, CTX, Amx and K
	23	V, E, C, R and Amp	G	P, CTX, Amx and K
	24	V, R and Amp	Amx	P, G, E, C, CTX and K
	25	G, C, R and Amp	E and K	P, V, CTX and Amx
	26	P, C, R and CTX	-	G, V, E, Amp, Amx and K
	27	P, V, C, R, Amp and K	E and Amx	G and R

Key: **P**- Penicillin, **C**- Chloramphenicol, **CTX**- Cefotaxime, **G**- Gentamycin, **E**- Erythromycin, **RD**- Rifampicin, **V**- Vancomycin, **Amp**- Ampicillin, **K**- Kanamycin and **Amx**- Amoxycilin

Table 8 below shows the occurrence and significance of each antibiotic tested against the three different sources. p- values were obtained by carrying out cross-tabulations and Chi-Square testing using SPSS V19.

No significant difference was found between the presence of *hlyA* or *inlB* between the three sample sites (p= 0.206). As tetracycline was tested by PCR, presence of tetracycline across all sources was generally low (12.9%) and 87.1% absent.

Table 8: Significance of antibiotic resistance profiles from the three different sources.

	Source						
Antibiotic	**LM**	**F**	**RS**	**Chi-Square Value**	**df**	**p-value**	**Significance**
tet(S)	86% S 14% R	84% S 16% R	92.5% S 7.5% R	1.524	2	0.467	No
P	100% R	80% R 20% S	50% R 50% S	6.042	2	0.05	Yes
G	10%R 20% I 70% S	20% R 10% I 70% S	16.7% R 16.7% I 66. 7% S	0.674	4	0.954	No
E	40% R 44.4% I 15.4% S	40% R 44.4% I 15.4% S	16.7% R 16.7% I 66.7% S	1.142	4	0.888	No
V	80% R 20% S	70% R 10% I 20% S	66.7% R 0% I 33.3% S	2.068	4	0.723	No
Cl	50% R 10% I 40% S	70% R 20% I 10% S	33.3% R 33.3% I 33.3% S	3.838	4	0.428	No
R	60% R 40% S	80% R 20% S	66.7% R 33.3% S	0.963	2	0.618	No

CTX	30% R 70% S	40% R 60% S	33.3% R 66.7% S	0.227	2	0.893	No
Amp	100% R	70% R 20% I 10% S	66.7% R 33.3% S	7.594	4	0.108	No
Amx	60% R 40% S	50% R 50% S	33.3% R 66.7% S	1.067	2	0.587	No
K	20% R 30% I 50% S	30% R 20% I 50% S	0% R 16.7% I 83.3% S	3.004	4	0.557	No

Key: **P**- Penicillin, **C**- Chloramphenicol, **CTX**- Cefotaxime, **G**- Gentamycin, **E**- Erythromycin, **RD**- Rifampicin, **V**- Vancomycin, **Amp**- Ampicillin, **K**- Kanamycin and **Amx**- Amoxycilin, **LM**- Local Markets, **F**- Poultry Farms and **RS**- Retail Stores.

From the ten antibiotics used, a total of six classes of antibiotics had been profiled. These classes were; macrolides, glycopeptides, aminoglycosides, β-lactams, chloraphenicols and rifamycins.

Table 9 below represents a summary of all *Listeria* isolates resistance to each of the antibiotics tested using Kirby-Bauer Disc Diffusion method. Highest resistance is seen in penicillin and ampicillin. The only statistically significant resistance was that of penicillin were as the others did not provide a p-value lower than 0.05. Again, percentages appear high due to the fact that from a total of 148 isolates, only 27 in total were *Listeria* species.

Table 9: Summary of percentage resistance to antibiotics among all *Listeria* isolates**.**

Antibiotic	**Percentage resistant of confirmed *L. monocytogenes***	**Percentage resistant of Presumptive *Listeria* species**	**Total**
Penicillin	93	39	81
Vancomycin	79	39	74
Rifampicin	64	43	70
Chloramphenicol	36	43	56
Cefotaxime	43	13	33
Ampicillin	79	48	81
Amoxicillin	7	-	4
Kanamycin	7	13	15
Gentamycin	14	9	15
Erythromycin	29	22	33

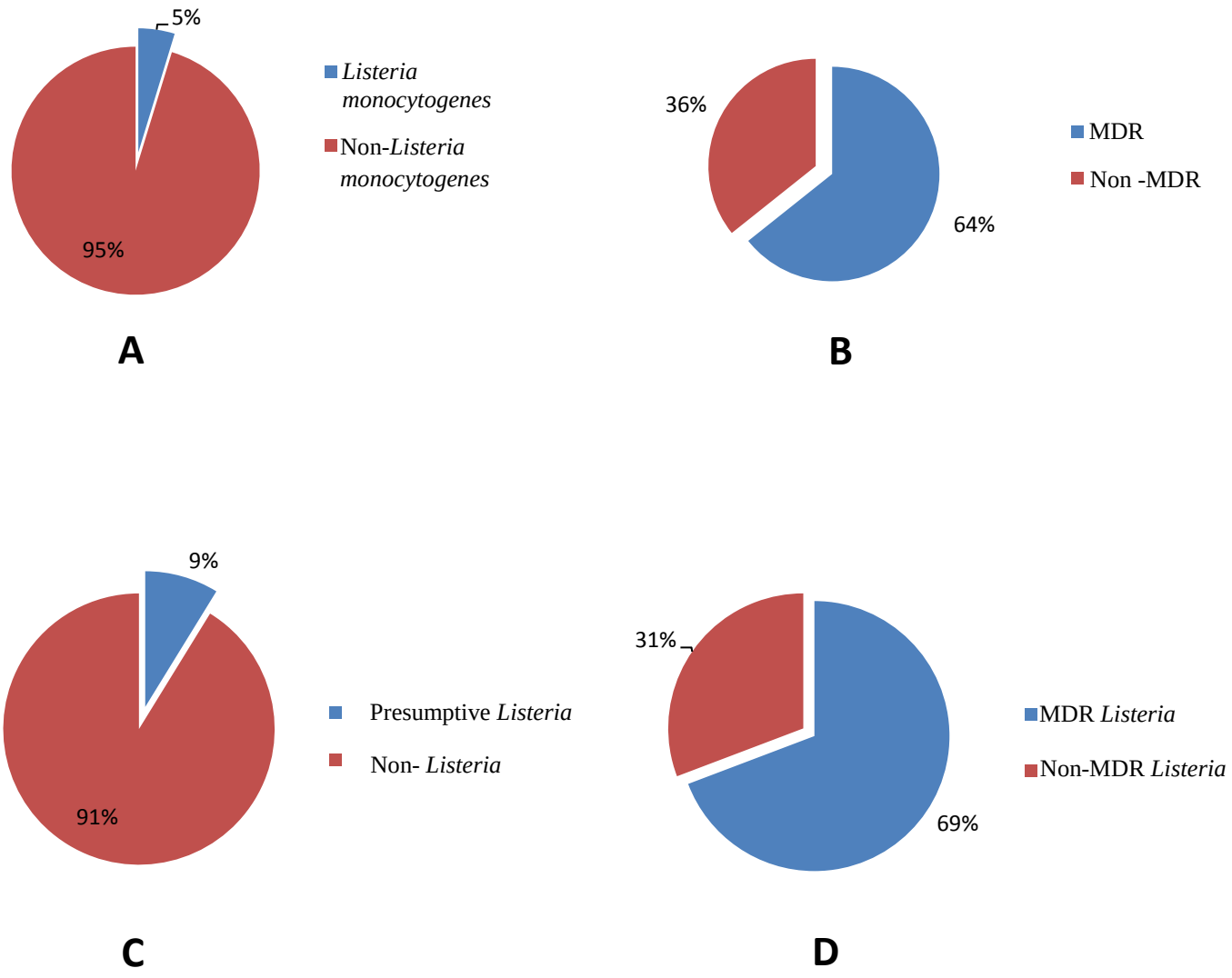

Figure 15: **A**- Percentage *L. monocytogenes* from all samples. **B**- Percentage Multi-drug resistant *L. monocytogenes*, **C**- Percentage presumptive *Listeria* from total samples, **D**- Percentage Multi-drug resistant presumptive *Listeria* from total presumptive *Listeria*.

CHAPTER 5

5 DISCUSSION

5.1 Detection of *Listeria monocytogenes* in chicken samples

A series of pre-enrichment broths, cultivation broths and selective agar were used to detect the presence of *L. monocytogenes* in the chicken samples. Tryptic Soy Broth (TSB) was used for pre-enrichment. Al-Zeyara *et al*., 2010 conducted a study comparing various different pre-enrichment broths such as TSB, Half Fraser Broth (HFB) and Oxoid Novel Enrichment Broth (ONE). From these broths, TSB performed the best for viable bacterial count across a range of food samples. Thereafter, incubation was done in *Listeria* Enrichment Broth Base with the addition of the SRO142E supplement provided by Oxoid. This broth was chosen as it has been prescribed by many studies done on *L. monocytogenes* (Nakarmura *et al*., 2005; Nicholson *et al*., 2005).

Inoculation from these broths was done on *Listeria* Selective Agar Base (LSA) supplemented with SRO140E provided by Oxoid. Positive *L. monocytogenes* colonies were visible as a brown colony with aesculin hydrolysis ("white halo"). Individual colonies were then used in DNA extraction and further downstream application of PCR for both the virulence genes as well as the tetracycline amplification.

5.2 Prevalence of *Listeria monocytogenes*

In the present study, *L. monocytogenes* contamination was at 9% from a total of 148 poultry samples. This is lower than that reported in a study done in Gauteng by Nierop *et al*., 2005, of 19% from 99 chicken carcasses. From the total of 14 positive *L. monocytogenes*, 7 were positive for the *hlyA* gene and 7 positive for *inlB* gene. For this study, the PCR protocol used the primers as described by Usman *et al*., 2016, for the amplification of the *hlyA* gene. These primers were then chosen as the best primer pair as they are designed to amplify specific fragments of *L. monocytogenes* which are both genetically and biochemically assessed to belonging to this species, producing a 702bp fragment. The primer set for the amplification of the *inlB* gene was previously used in a study conducted by Kim and Bhunia. (2008). The successful amplification of the *inlB* gene was obtained in their study under various growth conditions with detection of *L. monocytogenes* alongside *Salmonella enterica* and *Escherichia coli* 0157:H7. The *hlyA* gene encodes listerolysin O which is a major virulence factor and is responsible for the escape of the host cell vacuole (Seveau, 2014). When testing the virulence of *L. monocytogenes*, many researchers often use the presence of the *hlyA* gene as suitable confirmation (Dramsi and Cossart., 2002;

Seveau, 2014; Jamali *et al*., 2015; Kumar *et al*., 2016). Therefore, in this study the protocol described by Usman *et al*., 2016, gave positive amplification for the 7 *hlyA* isolates.

The *inlA* gene is responsible for a surface protein together with the *inlB* gene which aids in adhesion of *L. monocytogenes* to the infecting host cell (Chen *et al*., 2009). This provided a good basis to use the amplification of the *inlB* gene as the second virulence factor. Following the protocol described by Kim and Bhunia (2008), 7 isolates tested positive for the *inlB* gene.

An interesting finding in this study is that neither of the *hlyA* or *inlB* isolates amplified together. This is unconventional as the *hlyA* gene is found within a virulence gene cluster controlled by a positive regulatory factor PrfA. PrfA is also involved in the regulating the internal genes, *inlA*, *inlB* and *inlC* (Portnoy *et al*., 1992; Ryser., 1999; Jacquet *et al*., 2002).

The rationale behind this is that both *hlyA* and *inlB* alone may be sufficient to gain entry into the host cell (Jacquet *et al*., 2002).

Although rare, the occurrence of *L. monocytogenes* in poultry and broiler carcasses has been covered globally. A study done by Rorvik *et al*., 2003, in Norway reported *L. monocytogenes* occurrence as 75% in broiler carcasses, 48% in broiler cuts and 1% in both processed and grilled broilers. The 9% occurrence of *L. monocytogenes* in this study is a cause of concern as *L. monocytogenes* is zoonotic and it is likely that transmission from ruminant species to poultry will increase over time (Oevermann *et al*., 2010). Studies have also reported the occurrence of *L. monocytogenes* as 32% in conventional broiler flocks (Chemaly *et al*., 2008), 20.6%-24.1% in RTE poultry across two poultry processing plants (Gudbjörnsdóttir *et al*., 2004) and 8.9% from a poultry abattoir (Bouyard *et al*., 2015). There may also be possible *L. monocytogenes* isolates within the samples that do not display the *hlyA* gene. These *L. monocytogenes* may lack the *hlyA* gene due to being from the environment or lacking one or more virulence determinants as a result of spontaneous mutations (Usman *et al*., 2016). As seen in Table 5, the highest percentage of presumptive *Listeria* species was obtained from local markets (LM). This may be due to these areas not having a formal structure for hygiene control. Also it is important to note that most of these birds are grown without the use of antibiotics or supplements and kept in cramped broiler houses allowing for the spread and proliferation of *L. monocytogenes.* Retail stores (RS) showed the lowest occurrence of presumptive *Listeria* species as the broilers are obtained from farms that adhere to strict hygiene practices. Interestingly the highest percentage of confirmed *L. monocytogenes* isolates

were obtained from broiler farms (F) where the birds are believed to be treated with some kind of pathogen and disease control treatment. These farms are large in size as well as in stock of birds, therefore it can be speculated that the transmission of *L. monocytogenes* may be relative easy to the living conditions.

5.3 Antibiotic susceptibility testing of *L. monocytogenes*

From a total of 148 samples used in this study, 14 were positively identified as *L. monocytogenes*. These 14 *L. monocytogenes* were first screened for the presence of the *tet*(S) gene. This gene infers the presence of tetracycline resistance which belongs to the tetracycline antibiotic class. The percentage of isolates being positive for the tetracycline (S) gene was 12.9% which is considerably low. Thereafter, antibiotic susceptibility profiles were constructed using the Kirby-Bauer Disc Diffusion method. A total of 10 antibiotics were used which embodied 7 classes of antibiotics. From the 10 antibiotics used, 64% of *L. monocytogenes* were resistant to more than 3 classes of antibiotics. This therefore, placed them under the category of Multi-Drug Resistant *L. monocytogenes* in context of this study.

The most common antibiotic resistance was observed with penicillin (93%) followed by ampicillin and vancomycin (79%) with rifampicillin resistance (64%) as the third most common antibiotic. One isolate was recorded resistant to each amoxicillin and kanamycin which is a positive outcome as amoxicillin is used as the first line of treatment against listeriosis infections (Guerrero *et al*., 2011 and Chan *et al*., 2013). Resistance against chloramphenicol, cefotaxime, gentamycin and erythromycin was recorded at 36%, 43%, 14% and 29% respectively. One may argue that the resistance observed in this study is considerably high; however, it is important to note that the total number of positive *L. monocytogenes* was 14 isolates. Statistical analyses showed only penicillin resistance being significant (p-value = 0.05), while the other 9 antibiotics were not statistically significant (p-value>0.05).

A point of concern is raised when considering that 13 out of the 14 *L. monocytogenes* isolates showed resistance to penicillin. The reason for concern is that penicillin, amoxicillin and ampicillin are the three main antibiotics used in the treatment of listeriosis during pregnancy (Janakiraman, 2008). The dosage of these drugs are crucial for treatment however, it is generally accepted that high doses of these drugs are administered in order to ensure accurate penetration into the umbilical

cord and placenta (Janakiraman, 2008). The continuous use of penicillin and ampicillin may drive resistance in *L. monocytogenes* even higher than currently reported rendering its use ineffective. The use of vancomycin was also reported by Janakiraman (2008), to treat listerial meningitis, reporting that resistance to vancomycin is rare, the complete contrast is seen in this study.

5.4 Antibiotic susceptibility testing of presumptive *Listeria spp*.

Presumptive *Listeria spp*. were considered in this study as these did not amplify via PCR however, showed strong resemblance to typical *Listeria* colonies when grown on *Listeria* Selective Agar (LSA). Thirteen isolates were considered to be presumptive *Listeria* species and were also tested for the presence of the *tet*(S) gene and were subjected to the antibiotic resistant profiling. Tetracycline resistance showed 9 isolates out of 13 (69%) were positive, implying possible tetracycline resistance. The *tet*(S) gene shows 79 and 72% amino acid identity with *tet*(M) and *tet*(O) respectively (Charpentier *et al*., 1993). This is higher than that of a study done by Jamali and Thong (2014) which reported tetracycline resistance in *L. monocytogenes* at 49.4% from milk products. Another study by Li *et al.,* 2016, reported very low percentage of tetracycline resistance (20.5%) in pork meat. It was also observed that 69% of the presumptive *Listeria* showed resistance to 3 or more classes of antibiotics, again falling under MDR in context of this study.

It was also observed that in both the *L. monocytogenes* isolates and presumptive *Listeria* isolates, the lowest observed resistance was against amoxicillin. In contrast to the *L. monocytogenes* isolates, the highest resistance for the presumptive *L. monocytogenes* isolates was observed for ampicillin (48%) as compared to penicillin (93%). The second highest resistance was observed for both rifampicin and chloramphenicol, both recorded at 43% followed by penicillin and vancomycin at 39%. The data on antimicrobial resistance obtained in this study suggest the importance of a continued surveillance of emerging antimicrobial resistance in *L. monocytogenes* to control the pathogen and ensure effective treatment of human listeriosis. A study (Chou *et al.,* 2006) has reported the possible association between *L. monocytogenes* isolates from fish products and human listeriosis. However, food products contaminated with *L. monocytogenes* remain a potential risk to human health through cross-contamination during food preparation.

Another key factor for consideration is the relationship shared between *L. innoccua* and *L. monocytogenes*. In this study, presumptive *L. monocytogenes* isolates were assumed *L. monocytogenes* due to their appearance of selective agar, showing typical *L. monocytogenes* colony formation. However, these isolates could be *L. innocua* species as it is known that *L. innocua* is

found more frequently and has a higher frequency of recovery than compared *to L. monocytogenes* (El-Malek *et al*., 2010). Gomez *et al*. (2014) stated that *L. innocua* contain a reservoir of antibiotic resistance genes that can be transferred to *L. monocytogenes*, using an example of a multi-drug resistant plasmid called pDB2011. In this plasmid, a resistance gene for trimethoprim was found that was 100% identical to dfrD in *L. monocytogenes* isolates. A summary of the antibiotic resistance for all *Listeria* isolates, confirmed by PCR and presumptive, is given in Table 7. Overall, highest significant resistance was against penicillin, followed by ampicillin. Amoxicillin resistance was the lowest across all samples at 3%.

It is also important to consider those samples which intermediate resistance to antibiotics as these isolates provides a greater threat to combating antibiotic resistant bacteria. These isolates have the potential to become resistant as well as the ability to transfer resistance genes to surrounding bacterial colonies (WHO, 2015). Factors leading to intermediate resistant bacteria not just confined to the overuse of antibiotics but may be due to environmental pressures which results in the persistence of these bacteria (Howden *et al*., 2014).

5.5 Multiple drug resistance and antibiotic classes

5.5.1 Tetracyclines

L. monocytogenes and presumptive *Listeria* isolates were tested for the presence of the *tet*(S) gene. Positive *tet*(S) amplification was 42.8% and 84.6% for *L. monocytogenes* and presumptive *Listeria spp* respectively. Similar studies reported tetracycline resistance in *L. monocytogenes* at 37.5% (Garedew *et al*., 2015), 3.9% (Gomez *et al*., 2014) and 15.6% (Jamali and Thong., 2014). Across all isolates, 12.9% showed the presence of tetracycline and 87.1% absence of tetracycline. These however, did show any statistical significance ($P<0.05$) which may be due to the small sample size of 27 isolates. Previous studies done on *L. monocytogenes* and *L. innocua* have varied percentages of tetracycline resistance. Most of these studies looked into RTE meats or meat processing plants whereas other focused on retail raw meats as well as poultry processing plants. Percentages of tetracycline resistance were; 15.6% (Yan *et al*., 2010), 0.5% for *L. monocytogenes* and 9.2% for *L. innocua* (Gomez *et al*., 2014), 4.5% (Wang *et al*., 2013), 12.1% (Wang *et al*., 2015) for the RTE meats and meat processing plants. Poultry plants and retail foods reported resistance against tetracycline as 3% (Lyon *et al*., 2008) and 6.3% (Walsh *et al*., 2001). Although the presence of the

tetracycline (S) is considerably high for a small sample size, the actual resistance against tetracycline cannot be determined by using PCR.

5.5.2 Glycopeptides

L. monocytogenes isolates resistant against glycopeptides was recorded at 21% and presumptive *Listeria* at 23%. Overall, resistance to vancomycin was 73.1% with intermediate resistance at 3.8%. Vancomycin was used from this class of antibiotic as the resistance of vancomycin has been reported by many previous studies. These studies include resistance at 21% vancomycin resistance is pork meat (Doménech *et al*., 2015), 20.9% in seafood samples (Fallah *et al*., 2013) however, susceptible to vancomycin in a study done by Jamali and Thong (2014). Low resistance against vancomycin was reported by Counter *et al*. (2009) (0.8%), Wang *et al*. (2013) (5.4%), Walsh *et al*. (2001) (0.2%). In accordance with this study, *L. monocytogenes* isolated from dairy based products reported vancomycin resistance at 26%. This study also reported considerably high resistance to many other antibiotics such as penicillin (90%), ampicillin (60%), gentamycin (93.34%) and erythromycin (73.3%). This high level of resistance *in L. monocytyogenes* is of concern as vancomycin treatment is used in the final stages of human listeriosis treatment and therefore, further investigation into the glycopeptide resistance mechanism is needed (Fallah *et al*., 2013; Harakeh *et al*., 2009).

5.5.3 Macrolides

L. monocytogenes isolates resistant against erythromycin was recorded at 78% and presumptive *Listeria* at 69%. Overall, resistance against erythromycin was 34.6% with intermediate resistance at 11.5% however this was not regarded as statistically significant. This is very high when compared to erythromycin resistance in other studies such as 7.1% (Doménech *et al*., 2015), 2.52% (Fallah *et al*., 2013) and 7.2% (Jamali *et al*., 2014). These high numbers may also be due to the presence of *L. innocua* species within the presumptive *Listeria* isolates and have been reported to have higher resistance to many antibiotics as compared to *L. monocytogenes* (Walsh *et al*., 2001) Although *L. monocytogenes* has had resistance to erythromycin for quite some time, the multiple drug resistant strains, especially those resistant to gentamycin and erythromycin, are of concern as both these antibiotics are commonly used in the early treatment of human listeriosis infections (Byrne *et al*., 2016).

5.5.4 Amphenicols

L. monocytogenes isolates resistant against chloramphenicol was recorded at 14% and presumptive *Listeria* at 23%. Overall, resistance was at 53.8% and intermediate resistance at 19.2%. Complete susceptibility to chloramphenicols have been reported in a study done by Adzitey *et al*., 2013 and 0.1% in a study done by Walsh *et al*., 2001. In line with this study, a study done by Wang *et al*. (2015) reported chloramphenicol resistance at 33.3% with samples being isolated from RTE meat products. A combined resistance of chloramphenicol, gentamycin and erythromycin was reported at 41% in a study done by Aase *et al*., 2000, whereas in this study a similar combined resistance was noted at 33.7%. Recently a study released by El-Bhana *et al*., 2016, reported 9% resistance to chloramphenicols and 10.8% resistance in *Listeria* spp. with 27.8% resistance in *L. monocytogenes* (Jamali and Thong., 2014). Chloramphenicols have been used to treat *Listeria* infections together with erythromycin, tetracycline and rifampicin as a secondary does of antimicrobials (Camejo *et al*., 2011). Therefore, as the resistance of chloramphenicol in this study may be considered as intermediate, it is worrying as increasing resistance in chloramphenicols will lead to a drawback in human listeriosis infection treatments.

5.5.5 Rifamycin

L. monocytogenes isolates resistant against rifampicin was recorded at 7% and presumptive *Listeria* at 30%. Overall resistance to rifampicin was 69.2%. Studies reported resistance of rifampicin at 1% (Fallah *et al*., 2013), 0% (Jamali and Thong, 2014) and 1.6% (Counter *et al*., 2009) for *L. monocytogenes*. Rifampicin is used during a combination of drugs to treat listeriosis infection. Such was reported by Scheld (1983) in a rabbit model of meningitis, addition of rifampicin was equivalent to that ampicillin. Other studies also report low resistance to rifampicin such as 1.6% (Counter *et al*., 2009). Dairy isolates showed a relatively high resistance to rifampicin in a study done by Srinivasen *et al*., 2005 of which 84% were resistant. It is therefore crucial to investigate the possible rise of rifampicin resistance in *Listeria* species and to investigate the resistance mechanism in more depth.

5.5.6 *β*-lactams

L. monocytogenes isolates resistant against *β*-lactams was recorded at 57% and presumptive *Listeria* at 53%. Overall resistance to β-lactam in this study was 31.1% which is not statistically significant. This is however due to high resistance of cefotaxime and ampicillin which was 57.7% resistance in *L. monocytogenes* and 69% for presumptive *Listeria spp*. Fifty percent of the isolates were

susceptible to amoxicillin which is in accordance to the study done by Adzitey *et al*., 2013. However there has also been studies which reported fairly high resistance to ampicillin; 56%, 38.5%, 14.5% and 1.98% for El-Bhana *et al*., 2016, Fallah *et al*., 2013, Jamali and Thong., 2014 and Walsh *et al*., 2001 respectively.

5.5.7 Aminoglycosides

L. monocytogenes isolates resistant against aminoglycosides was recorded at 50% and presumptive *Listeria* at 53%. Overall aminoglycoside resistance was reported at 17.3%. The aminoglycoside class consisted of two antibiotics, gentamicin and kanamycin. Gentamicin resistance for *L. monocytogenes* was 28.5% and kanamycin at 14.28%. Previous studies reported gentamycin resistance between 0% and 0.72% (Walsh *et al*., 2001; Counter *et al*., 2009; Pesavento *et al*., 2010; Fallah *et al*., 2013; Jamali and Thong., 2014). El-Bhana *et al*., 2016 reported gentamycin resistance as high as 48%. Kanamycin resistance is usually reported in low counts as well, anywhere between 0% and 3% (Wong *et al*., 1990; Jamali and Thong., 2014; Chen *et al*., 2014). It is however known that the use and misuse of antibiotics over the years have led to an increase in resistance to these antibiotics (Aureli *et al*., 2003).

CHAPTER 6

6 CONCLUSION AND RECOMMENDATIONS

6.1 Overall occurrence of *L. monocytogenes* and its implications

In conclusion, the results obtained in this study may be useful in re-looking at the current treatment of human listeriosis. Nine percent occurrence in *L. monocytogenes* obtained in this study is considerably high due to the small sample pool used. The occurrence of the *hlyA* gene and *inlB* gene was 4.7% indicating that the totals of 14 positive *L. monocytogenes* isolates contained virulence factors and it may be possible to cause infection in humans. Although *L. monocytogenes* may be found in ruminant animal droppings, it is generally accepted that infections with this organism is most likely caused by ingestion of contaminated food products. It is however important to note that the transmission of this organism from one animal species to another can be passed via feces (Nwachukwu and Orji, 2012). Mendonca *et al*., 2015 reported contamination of frozen chicken packaged and ready to sell with *L. monocytogenes* at 33%. Kosek-Paszkowska *et al*., 2005 reported only 1% *L. monocytogenes* isolated in raw poultry meat with majority of the *Listeria spp* being *L. innocua*. Currently the treatment of choice for this infection is the use of ampicillin, rifampicin and penicillin with the addition of gentamicin, however, many studies have now reported a few incidences of resistance to these antimicrobials. An important factor to consider is the treatment of those whose immune systems have been comprised by existing disease such as HIV/AIDS and pregnant women. In these cases the treatment regime changes to the use of vancomycin and erythromycin which now also begin to show resistance in *L. monocytogenes*. It also noted that the source from which broilers are obtained can play a crucial role in the occurrence of *L. monocytogenes* as seen in this study.

This is important to consider as *L. monocytogenes* may be transmitted to other foods from poultry which then may be ingested causing listeriosis. As previously stated, the control and management of *Listeria* and other disease causing organisms is still relatively weak in South Africa as compared to first world countries (FAO/WHO Regional Conference, October 2005). Therefore the implication of the presence of *L. monocytogenes* in food broiler chickens, regardless of the percentage, poses a threat as most of these broilers especially those obtained from the local markets are being sold to South Africans who do not have easy access to clinics and doctors in order to seek treatment.

6.2 Overall Resistance in *L. monocytogenes* and *Listeria spp*.

It also known that *L. monocytogenes* is able to transfer plasmids with resistant genes be it in vitro or in vivo in the intestinal tract as well as in response to environmental stresses (Liang *et al*., 2016). This therefore, provides an opportunity for other species of *Listeria* such as *Listeria innocua* to gain resistance to commonly used antimicrobials. Overall resistance by *L. monocytogenes* may be consider high. The highest resistance was observed in penicillin, vancomycin and rifampicillin. This has a serious implication for South African studies as penicillin G is used in the first line of listeriosis treatment (Charpentier *et al.*, 1999). Dan *et al*., 2015 reported in their study of *L. monocytogenes*, the highest resistance was against tetracycline and chloramphenicol. In this study resistance against tetracycline was 42.8% for *L. monocytogenes* and 84.6% in other *Listeria* spp. Using the resistance profiles obtained in this study together with the work done previously, it can be deduced that antimicrobial resistance in all *Listeria* species are going to rapidly increase over time. It is however known that the use and misuse of antibiotics over the years have led to an increase in resistance to these antibiotics (Aureli *et al*., 2003). Also noting that 64% of the *L. monocytogenes* were resistant to multiple antibiotics, South African health care systems may consider a re-evaluation of the antibiotics being used in animal feed and treatment as well as human treatment of listeriosis. It can therefore be concluded that treatment against listeriosis should be a synergistic effect of using both ampicillin and gentamycin (Walsh *et al*., 2001), with gentamycin resistance in this study at 14% for *L. monocytogenes*. With the dissemination and development of antimicrobial resistance in animal production still remaining unclear it would be advised that continual surveillance and monitoring of these mass production houses are vital in order to curb the rise of listeriosis infection (Morobe *et al*., 2012).

6.3 Recommendations

Although this study proved significant in its findings of high resistance to the most commonly used antibiotics to treat listeriosis infections, further work in this field will provide an even greater understanding of the seriousness of antibiotic resistance in *L. monocytogenes*. The sample size used for this study consisted of 148, where only 27 isolates were used for further testing. Knowing that *Listeria* species is not common in poultry, further studies on this topic should consider the use of a larger sample size. The geographical locations of which samples were collected boarded the Durban area however, there are many farms which provide poultry to local citizens and small vendors inland, which if sampled, would provide a greater depth of the study. The virulence genes tested for in this study provided a reliable basis for detecting *L. monocytogenes* however the more virulence

genes tested against, the better the understanding of the virulence of the organism. Virulence genes which could be used in further studies could be the VirR gene, PrfA, PrsA2 and ActA. In this study, it was unable to clearly differentiate isolates which were not *L. monocytogenes*, it is therefore recommended that differentiation of the presumptive isolates would add to the occurrence of other *Listeria* species within broiler ceaca. Finally, the resistance of *L. monocytogenes* will need to be further studied in order to provide a greater understanding in the high resistance profiles seen in this study. Also the acquisition of these resistance genes will need to be considered as there is an increase in antibiotic resistance over the years till current.

References

Aasea, B., Sundheimb, G., Langsrudb, S. and Rørvika, L. M. (2000). Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes. International Journal of Food Microbiology*. **62**, 57-63.

Abuin, F. C. M., Fernandez, Q. E., Sampayo, F. C., Otero,. R. J. L., Rodriguez, D. L. and Cepeda S. (1994). Susceptibility of *Listeria* Species Isolated from Food to Nine Antimicrobial Agents. *Antimicrobial Agents and Chemotherapy* **38**, 1655-1657.

Adzitey, F., Ali, G. R. R., Huda, N., Cogan, T. and Corry, J. (2013). Prevalence, antibiotic resistance and genetic diversity of *Listeria monocytogenes* isolated from ducks, their rearing and processing environments in Penang, Malaysia. *Food Control*. **33**, 607-614.

Aguado, V., Vitas, A. I. and Garcia- Jalon, I. (2004). Characterization of *Listeria monocytogenes* and *Listeria innocua* from vegetable processing plant by RAPD and REA. *International Journal of Food Microbiology*. **90**, 341-347.

Aldred, K. J., Kerns, R. J., and Osheroff, N. (2014). Mechanism of quinolone action and resistance. *Biochemistry*. **53**(10), 1565-1574.

Al-Ghamdi, M., Al-Mustafa, Z., El-Morsy, F., Al-Faky, A., Haider, I. and Essa, H. (2000). Residues of tetracycline compounds in poultry products in the eastern province of Saudi Arabia. *Public Health*. **114**, 300-304.

Allerberger, F., and Wagner, M. (2010). Listeriosis: a resurgent foodborne infection. *Clinical Microbiology and Infection*, **16**(1), 16-23.

Al-Zeyara, S. A., Jarvis, B. and Mackey, B. M. (2010). The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broths. *International Journal of Food Microbiology*. **145**, 98-105.

Anadon, A. (2006). The EU Ban of Antibiotics as Feed Additives: Alternatives and consumer safety, in: Workshop III: 2006 EU Ban on Antibiotics as Feed Additives: Consequences and Perspectives. *Journal of Veterinary Pharmacology and Therapeutics*. **29**, 41-46.

Aureli, P., Ferrini, A. M., Mannoni, V., Hodzic, S., Wedell-Weergaard, C., and Oliva, B. (2003). Susceptibility of *Listeria monocytogenes* isolated from food in Italy to antibiotics. *International journal of food microbiology*, **83**(3), 325-330.

Balizs, G. and Hewitt, A. (2003). Determination of veterinary drug residues by liquid chromatography and tandem mass spectrometry. *Analytica Chemica Acta*. **492**, 105-131.

Baloga, A. O. and Harlander, S. K. (1999). Comparison of Methods for Discrimination between Strains of *Listeria monocytogenes* from Epidemiological Survey. *Applied Environmental Microbiology*. **57**, 2324-2331.

Barile, S., Devirgiliis, C., and Perozzi, G. (2012). Molecular characterization of a novel mosaic tet (S/M) gene encoding tetracycline resistance in foodborne strains of *Streptococcus bovis*. *Microbiology*. **158**(9), 2353-2362.

Bedford, M. (2000). Removal of antibiotic growth promoters from poultry diets: implications and strategies to minimise subsequent problems. *World's Poultry Science Journal*. **56**, 347-365.

Bekondi, C., Bernede, C., Passone, N., Minssart, P., Kamalo, C., Mbolidi, D., Germani, Y., (2006). Primary and opportunistic pathogens associated with meningitis in adults in Bengui, Central Africa Republic, in relation to human immunodeficiency virus serostatus. *International Journal Infectious Diseases*. **10**, 387-95.

Bell, C., and Kyriakides, A. (1998). Factors affecting the growth and survival of *Listeria monocytogenes*. In *Listeria* (30-34). Springer US.

Bille, J., Catimel, B., Bannerman, E., Jacquet, C. Yersin, M. N., Caniaux, I. Monget, D. and Rocourt, J., (1992). API *Listeria*, a new and Promising One –Day System To Identify *Listeria* Isolates. *Journal of Applied and Environmental Microbiology.* **58**, 1857- 1860.

Bouayad, L., Hamdi, T. M., Naim, M., Leclercq, A., and Lecuit, M. (2015). Prevalence of *Listeria spp*. and Molecular Characterization of *Listeria monocytogenes* Isolates from Broilers at the Abattoir. *Foodborne pathogens and disease*. **12**(7), 606-611.

Brizio, A. P. D. R. and Prentice, C. (2015). Chilled broiler carcasses: a study on the prevalence of *Salmonella*, *Listeria* and *Campylobacter*. *International Food Research Journal*, **22**(1), 55-58.

Brooks, J. P., McLaughlin, M. R., Adeli, A. and Miles, D. M. (2015). Pathogen re-colonization of in-house composted and noncomposted broiler litter. *The Journal of Applied Poultry Research*, **24**(2), 157-167.

Bruhn, J. B., Vogel, B. F. and Gram, L. (2005). Bias in the *Listeria monocytogenes* Enrichment Procedure: Lineage 2 Strains Outcompete Lineage 1 Strains in University of Vermont Selective Enrichments. *Applied and Environmental Microbiology*. **71**(2), 961-967.

Buchanan R. L, Golden, M. H., and Whiting, R. C. (1993). Differentiation of the effects of pH and lactic acid or acetic acid concentration on the kinetics of *Listeria monocytogenes* inactivation. *Journal of Food Protection* **56**, 474-478.

Buchrieser, C. (2007). Biodiversity of the species *Listeria monocytogenes* and the genus *Listeria*. *Microbes and Infection*. **9**, 1147-1155.

Bush, K. A. R. E. N. (1989). Characterization of beta-lactamases. *Antimicrobial Agents and Chemotherapy*. **33**(3), 259-263.

Byarugaba, D. K. (2004). A view on antimicrobial resistance in developing countries and responsible risk factors. *International Journal of Antimicrobial Agents*. **24**, 105-110.

Byrne, V. V., Ernesto, H., Vallim, D. C. and Comastri de Castro, A. R. (2016). Occurrence and antimicrobial resistance patterns of *Listeria monocytogenes* isolated from vegetables. *Brazilian Journal of Microbiology*. **47**(2), 438-443.

Camejo, A., Carvalho, F., Reis, O., Leitão, E., Sousa, S., and Cabanes, D. (2011). The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. *Virulence*. ***2***(5), 379-394.

Castanon, J. I. R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science*. **86**, 2466-2471.

Chan, B. T., Hohmann, E., Barshak, M. B., and Pukkila-Worley, R. (2013). Treatment of Listeriosis in First Trimester of Pregnancy . *Emerging Infectious Diseases*, **19**(5), 839–841.

Charpentier, E. and Courvalin, P. (1999). Antibiotic resistance in *Listeria* species. *Antimicrobial Agents and Chemotherapy*. **43**, 2103-2108.

Charpentier, E., Gerbaud, G., Jacquet, C., Rocourt, J., and Courvalin, P. (1995).Incidence of antibiotic resistance in *Listeria* species. *Journal of Infectious Diseases* **172**, 277–281.

Chemaly, M., Toquin, M. T., Le Nôtre, Y., and Fravalo, P. (2008). Prevalence of *Listeria monocytogenes* in poultry production in France. *Journal of Food Protection*. **71**(10), 1996-2000.

Chen, B. Y., Kim, T. J., Juan, L., Silva, J. L. and Jung, Y. S. (2009). Positive Correlation Between the Expression of *inlA* and *inlB* Genes of *Listeria monocytogenes* and Its Attachment Strength on Glass Surface. *Food Biophysics*. **4**, 304-311.

Chen, J., Brosch, R. and Luchansky, J. B. (2006). Isolation and Characterization of *Listeria monocytogenes* – Specific Nucleotide Sequences. *Journal of Applied and Environmental Microbiology*. **59**, 4367-4370.

Cheng, L., Yuan, T. and Zhang, Y. (2003). Rapid Preparation of Total Nucleic Acids from *E. coli* for Multi-purpose Applications. *Journal of Biochemistry and Microbiology*. **37**(3), 351-355.

Chiara, M., Caruso, M., D'Erchia, A. M., Manzari, C., Fraccalvieri, R., Goffredo, E., Latorre, L., Miccolupo, A., Padalino, I., Santagada, G., Chiocco, D., Pesole, G., Horner, D. S. and Parisi, A. (2015). Comparative genomics of *Listeria sensu lato*: genus-wide differences in evolutionary dynamics and the progressive gain of complex, potentially pathogenicityrelated traits through lateral gene transfer. *Genome Biology Evolution* **7**, 2154–2172.

Chintu C. and Bathirunathan, N. (1975). Bacterial meningitis in infancy and childhood in Lusaka (One year prospective study). *Medical Journal of Zambia*.**9,** 150-7.

Chopra, I. and Roberts, M. (2001). Tetracycline Antibiotics: Mode of action, application, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*. **65**, 232-260.

Chou, C. H., Silva, J. L. and Wang, C. Prevalence and typing of *Listeria* monocytogenes in raw catfish fillets. J Food Prot 2006;69:815–819.

Cocolin L., Rantitsiou, K., Iacumin, L., Cantoni, C. and Comi, G. (2002). Direct identification in food samples of *Listeria* species and *Listeria monocytogenes* by molecular methods. *Journal of Applied and Environmental Microbiology*. **68**, 6273 -6282.

Conter, M., Paludi, D., Zanardi, E., Ghidini, S., Vergara, A. and Ianieri, A. (2009). Characterization of antimicrobial resistance of foodborne *Listeria monocytogenes*. *International Journal of Food Microbiology*. **128**, 497-500.

Corcia, A. and Nazzari, M. (2002). Liquid chromatographic-mass spectrometric methods for analyzing antibiotic and antibacterial agents in animal food products. *Journal of Chromatography, A*. **974**, 53-89.

Crim, S. M., Griffin, P. M., Tauxe, R., Marder, E. P., Gilliss, D., Cronquist, A. B. and Lathrop, S. (2015). Preliminary incidence and trends of infection with pathogens transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 US sites, 2006–2014. *MMWR Morb Mortal Wkly Rep*, **64**(18), 495-9.

Czajka, J., Bsat, N., Piani, M., Russ, W., Sultana, K., Weidmann, M ., Whitaker, R. and Batt, C. A. (1993). Differentiation of *Listeria monocytogenes* and *Listeria innocua* by 16s rRNA Genes and Intraspecies Discrimination of *Listeria monocytogenes* Strains by Random Amplified Polymorphic DNA Polymophism. *Journal of Applied and Environmental Microbiology*. **59**, 304-308.

Dahshan, H., Merwad, A. M. and Mohamed, T. S. (2016). *Listeria* Species in Broiler Poultry Farms: Potential Public Health Hazards. *Journal of microbiology and biotechnology*, **26**(9), 1551-1556.

Davies, J. E. (1997). Origins, acquisition and dissemination of resistance determinants. In Antibiotic resistance: Origins, evolution, selection and spread, edited by Anonymous John Wiley and Sons, **207**, 15-27.

Dayan, A. D. (1993). Allergy to antimicrobial residues in food: assessment of the risk to man. *Veterinary Microbiology*. **35**, 213-226.

Dewdney, J. M., Maes, L., Raynaud, J. P., Blanc, F., Scheid, J. P., Jackson, T., Lens, S. and Verschueren, C. (1991). Risk assessment of antibiotic residues of beta-lactams and macrolides in food products with regard to their immuno-allergic potential. *Food and Chemical Toxicology*. **29**, 477-483.

Dibner, J. J. and Richards, J. D. (2005). Antibiotic growth promoters in agriculture: history and mode of action. *Poultry Science*. **84**, 634-643.

Doménech, E., Jimenez-Belenguer, A., Amoros, J. A., Ferrus, M. A., and Escriche, I. (2015). Prevalence and antimicrobial resistance of *Listeria monocytogenes* and *Salmonella* strains isolated in ready-to-eat foods in Eastern Spain. *Food Control*. **47,** 120-125.

Donoghue, D. J. (2003). Antibiotic residues in poultry tissues and eggs: human health concerns?. *Poultry Science*. **82**, 618-621.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C. and Martin, P. (2004). Differentiation of the Major *Listeria monocytogenes* Serovars by Multiplex PCR. *Journal of clinical Microbiology*. **42**(8), 3819-3822.

Dramsi, S. and Cossart, P. (2002). Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite. *The Journal of Cell Biology*. **156**(6), 943-946.

Duffy, L.L.,Vanderlinde, P.B., and Grau, F.H. (1994).Growth of *Listeria monocytogenes* on vacuum-packed cooked meats; effects of low pH, aw, nitrite and ascorbate. *International Journal of Food Microbiology*. **23,** 77-390.

El-Banna, T. E. S., Sonbol, F. I., Zaki, M. E. S., and El-Sayyad, H. H. I. (2016). Clinical and Environmental Prevalence and Antibiotic Susceptibility of *Listeria monocytogenes* in Dakahlea Governorate, Egypt. *Clin Microbiol*. **5**(3), 1-8.

El-Malek**,** A. M. A., Fathi, S., Ali, H., Hassanein, R., Mohamed, M. A., and Elsayh, K. I. (2010). Occurrence of *Listeria species* in meat, chicken products and human stools in Assiut city,

Egypt with PCR use for rapid identification of *Listeria monocytogenes*. *Veterinary World*. **3**(8), 353-359.

Elmali, M., CAN, H. Y. and Yaman, H. (2015). Prevalence of *Listeria monocytogenes* in poultry meat. *Food Science and Technology (Campinas)*, **35**(4), 672-675.

Embarek, P.K.B. (1994). Presence, detection and growth of *Listeria monocytogenes* in seafood: a review. *International Journal of Food Microbiology* **23**, 17-34.

European commission. 2001. Establishment of maximum residue limits (MRLs) for residues of veterinary medicinal products in foodstuffs of animal origin. Volume 8, Notice to Applicants and note for guidance.

Fallah, A. A., Saei-Dehkordi, S. S., and Mahzounieh, M. (2013). Occurrence and antibiotic resistance profiles of *Listeria monocytogenes* isolated from seafood products and market and processing environments in Iran. *Food control*, **34**(2), 630-636.

FAO/WHO regional conference on food safety for Africa Harare, Zimbabwe, 3-6 October (2005). International, regional, subregional and national cooperation in food safety in Africa, Prepared by The WHO Regional Office for Africa, BP 06, Brazzaville, Republic of Congo.

Farber J. M. and Peterkin P. I. (1991). *Listeria monocytogenes*: a food-borne pathogen. *Microbiology Review* **55**, 476-511.

Farouk, F., Azzazy, H. M., and Niessen, W. M. (2015). Challenges in the determination of aminoglycoside antibiotics, a review. *Analytica chimica acta*, **890**, 21-43.

Finlay, B. B. (2001). Cracking *Listeria*'s password. *Science*. **292**, 1665-1667.

Ferreira, V., Wiedmann, M., Teixeira, P., and Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *Journal of Food Protection*, **77**(1), 150-170.

Gandhi, M., and Chikindas, M. L. (2007). *Listeria*: a foodborne pathogen that knows how to survive. *International journal of food microbiology*, **113**(1), 1-15

Garedew, L., Taddese, A., Biru, T., Nigatu, S., Kebede, E., Ejo, M. and Birhanu, T. (2015). Prevalence and antimicrobial susceptibility profile of *Listeria* species from ready-to-eat foods of animal origin in Gondar Town, Ethiopia. *BMC Microbiology*. **15**, 100.

Gilot, P., Genicot, A., and Andre, P. (1996). Serotyping and esterase typing for analysis of *Listeria monocytogenes* populations recovered from foodstuffs and from human patients with listeriosis in Belgium. *Journal of Clinical Microbiology*. **34**, 1007-1010.

Global Poultry Trends: Faster Chicken Uptake Outside the EU. The Poultry Site. Retrieved July, 14, 2016, from http://www.thepoultrysite.com/articles/2979/global-poultry-trends-2013-faster-chicken-uptake-outside-the-eu/

Gomez, D., Azon, E., Marco, N., Carraminana, J. J., Rota, C., Arino, A. and Yangüela, J. (2014). Antimicrobial resistance of *Listeria monocytogenes* and *Listeria innocua* from meat products and meat-processing environment. *Food Microbiology*. **42**, 61–65.

Gudbjornsdottir, B., Suihko, M. L., Gustavsson, P., Thorkelsson, G., Salo, S. and Sjoberg, A. M. (2004). The incidence of *Listeria monocytogenes* in meat, poultry and seafood plants in the Nordic countries. *Food Microbiology*. **21**, 217–225.

Gudmundsdottir, S. B., Gudbjörnsdóttir, H. L., Lauzon, L., Einarsson, H. Kristinsson, K.G., and Kristjănsson, M. (2005). Tracing *Listeria monocytogenes* cold - smoked salmon and its processing environment in Iceland using pulse-field electrophoresis. *International Journal of Food Microbiology*. **101**, 41-51.

Guerrero, M. L. F., Torres, R., Mancebo, B., Gonza´lez-Lo´ pez, J. J., Go´ rgolas, M., Jusdado, J. J. and Roblas, R. F. (2011). Antimicrobial treatment of invasive non-perinatal human listeriosis and the impact of the underlying disease on prognosis. *Clinical Microbiology Infections*. **18**, 690-695.

Hadorn, K., Hachler, H., Schaffner, A. and Kayser, F. H. (1993). Genetic characterization of plasmid-encoded multiple antibiotic resistance in a strain of *Listeria monocytogenes* causing

endocarditis. *European Journal of Clinical Microbiology and Infectious Diseases*. **12**, 928–937.

Harakeh, S., Saleh, I., Zouhairi, O., Baydoun, E., Barbour, E. and Alwan, N. (2009). Antimicrobial resistance of *Listeria monocytogenes* isolated from dairy-based food products. *Science of the Total Environment*. **407**, 4022-4027.

Henry, R. and Rothwell, G. (1995). Industry organization and structure, In: The World Poultry Industry. International Finance Corporation, The World Bank, Washington D.C., ISBN: 0-82123429-8.

Herd, M. and Kocks, C., (2001). Gene fragments distinguishing an epidemic associated strain from virulent prototype strain of *L. monocytogenes* belong to a distinct function subset of genes and partially cross-hybridize with other *Listeria* species. *Infection and Immunity*. **69**, 3972-3979.

Hernandez-Milian, A., & Payeras-Cifre, A. (2014). What is new in listeriosis?. *BioMed research international*, *2014*.

Hof, H., Nichterlein, T. and Kretschmar, M. (1998). Management of listeriosis, *Clinical Microbiology Reviews*. **10**, 345–357.

Höhne, K., Loose, B., and Seeliger, H. P. (1974, December). Isolation of *Listeria monocytogenes* in slaughter animals and bats of Togo (West Africa). In *Annales de microbiologie* .**126**(4), 501-507.

Howard, P. J., Harsono, K. D. and Luchansky J. B., (1992). Differentiation of *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii* and *Listeria seeligeri* by Pulsed – Field Gel Electrophorsis. *Journal of Applied and Environmental Microbiology*. **58**, 709-712.

Howden, B. P., Peleg, A. Y. and Stinear, T. P. (2014). The evolution of vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous-VISA. *Infection, Genetics and Evolution*, **21**, 575-582.

Hubbert, W. T., Hagstad, H. V., Spangler, E., Hinton, M. H. and Hughes, K. L. (1996). Food Safety and Quality Assurance, in Food of Animal Origin, second edition, Iowa State University Press/ Ames, Iowa, 50014. ISBN: 0-8138-0714-x.

Jackson, B. R., Tarr, C., Strain, E., Jackson, K. A., Conrad, A., Carleton, H. and Silk, B. J. (2016). Implementation of nationwide real-time whole-genome sequencing to enhance listeriosis outbreak detection and investigation. *Clinical Infectious Diseases*, **63**(3), 380-386.

Jacquet, C., Gouin, E., Jeannel, D., Cossart, P. and Rocourt, J. (20020. Expression of ActA, Ami, InlB, and listeriolysin O in *Listeria monocytogenes* of human and food origin. *Journal of Applied and Environmental Microbiology*. **68**, 616-622.

Jamali, H. and Thong, K. L. (2014) Genotypic characterization and antimicrobial resistance of *Listeria monocytogenes* from ready-to-eat foods. *Food Control*, **44**: 1-6.

Jamali, H., Paydar, M., Ismail, S., Looi, C. Y., Wong, W. F., Radmehr, B. and Abedini, A. (2015). Prevalence, antimicrobial susceptibility and virulotyping of *Listeria* species and *Listeria monocytogenes* isolated from open-air fish markets. *BMC Microbiology*. **15**(144), 1-7.

Janakiraman, V. (2008). Listeriosis in Pregnancy: Diagnosis, Treatment, and Prevention. *Reviews in Obstetrics and Gynecology*, **1**(4), 179–185.

Jesús Cortés-Sánchez, A., Díaz-Ramirez, M., Villanueva-Carvajal, A., de la Luz Sánchez-Mundo, M., & Garcia-Barrientos, R. (2016). Health Risk Derived From Foods, About *Listeria monocytogenes*. *Int. J. Pure App. Biosci*, **4**(5), 29-41.

Jin, S. (1997). Regulations, realities and recommendations on antimicrobial use in food animal production in China, in: The Medical Impact of the Use of Antimicrobial in Food Animals, section 2.3.4.Geneva: World Health Organization.

Jorgensen, J. H., and Turnidge, J. D. (2015). Susceptibility test methods: dilution and disk diffusion methods. In *Manual of Clinical Microbiology, Eleventh Edition* (pp. 1253-1273). American Society of Microbiology.

Jukes, T. H. (1955). Antibiotics in Nutrition, In: Antibiotics Monographs. Medical Encyclopedia Inc. New York. The University of California.

Jukes, T. H. and williams, W. L. (1953). Nutritional effects of antibiotics. *Pharmacological Reviews*. **5**, 381-420.

Kathariou, S. (2000). Pathogenesis determinants of *Listeria monocytogenes*, 295-314. In J. W. Cary, J. E. Linz, and D. Bhatnagar (ed.), Microbial foodborne diseases. Technomics Publishing Co., Inc., Lancaster, PA.

Kim, H. and Bhunia, A. K. (2008). SEL, a Selective Enrichment Broth for Simultaneous Growth of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. *Applied and Environmental Microbiology*. **74**(15), 4853-4866.

Kinney, N. and Robles, A. (1994). The effect of mixing antibiotics with Marek's disease vaccines. Western Poultry Diseases Conference, Sacramento, California, USA. **43**, 96-97.

Kirchner, M., and Higgins, D. E. (2008). Inhibition of ROCK activity allows InlF-mediated invasion and increased virulence of *Listeria monocytogenes*. *Molecular microbiology*, **68**(3), 749-767.

Koch, J., Dworak, R., Prager, R., Becker, B., Brockmann, S., Wicke, A., and Stark, K. (2010). Large listeriosis outbreak linked to cheese made from pasteurized milk, Germany, 2006–2007. *Foodborne Pathogens and Disease*, **7**(12), 1581-1584.

Kumar, V. B., Fairoze1, N. N., Madhavaprasad, C., Karabasanavar, N.,, Kotresh, A., Nadoor, P., Prashant, S., Shilpa, A., Barbuddhe, S. B., Kurkure, N. and Chaudhary, S. (2016). Molecular characterization and antibiogram of *Listeria monocytogenes* isolated from chicken and mutton of retail markets. *Journal of Microbiology and Infectious Diseases*. **6**(2), 65-68.

Lachica, V. (1990). Same – Day Identification Sheme for Colonies of *Listeria monocytogenes*. *Journal of Environment Microbiology*. **56**, 1166-1168.

Lambertz, S. T., Nilsson, C., Brådenmark, A., Sylvén, S., Johansson, A., Jansson, L. M., and Lindblad, M. (2012). Prevalence and level of *Listeria monocytogenes* in ready-to-eat foods in Sweden 2010. *International Journal of Food Microbiology*, **160**(1), 24-31.

Lammerding, A. M., Glass, K .A., Gendron – Fitzpatrick, A. and Doyle, M. P. (1992). Determination of Virulence of Different Strains of *Listeria monocytogenes* and *Listeria innocua* by Oral Inoculation of Pregnant Mice. *Journal of Applied and Environmental Microbiology*. **58**, 3991- 4000.

Law, W. A. and Payne, L. N. (1996). The Poultry Industry, in Poultry diseases, edited by F. T. W.Jordan and M. Pattison , *4th edition*, W. B. Saunders Co. Ltd.: London.

Lazaro, R., Hernandez, D., Scortti, M., Esteve, M., Vazquez-Boland, T. J. A., and Pla, M. (2004). Quantitative Detection of *Listeria monocytogenes* and *Listeria innocua* by Real- Time PCR: Assessment of *hly*, *iap* and *lin*02483 Targets and AmpliFluor Technology. *Journal of Applied and Environmental Microbiology*. **70**, 1366-1377.

Leclercq, R. (2002). Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. *Clinical Infectious Disease*. **34**, 482-492.

Leimeister-Wächter, M. and Chakraborty, T. (1989). Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*. *Infection and Immunity*. **57**, 2350-2357.

Levy, S. B. (1992). The antibiotic paradox. How miracle drugs are destroying the miracle. Plenum Publishing Corporation, NY, ISBN 0306443317.

Li, L., Olsen, R. H., Ye, L., Wang, W., Shi, L., Yan, H., and Meng, H. (2016). Characterization of Antimicrobial Resistance of *Listeria monocytogenes* Strains Isolated from a Pork Processing Plant and Its Respective Meat Markets in Southern China. *Foodborne pathogens and disease*. **13**(5), 262-268.

Low, J. C. and Donachie, W. (1997). A Review of *Listeria monocytogenes* and Listeriosis. *The Veterinary Journal*. **153**, 9-29.

Lyon, S. A., Berrang, M. B., Fedorka-Cray, P. J., Fletcher, D. L. and Meinersmann, R. J. (2008). Antimicrobial Resistance of *Listeria monocytogenes* Isolated from a Poultry Further Processing Plant. *Food Borne Pathogens and Disease*. **5**(3), 253-259.

Mabote, K. I., Mbewe, M., and Ateba, C. N. (2011). Prevalence of Campylobacter contamination in fresh chicken meat and milk obtained from markets in the North-West Province, South Africa. *Journal of Human Ecology*, **36**(1), 23-28.

Malhotra-Kumar, S., Lammens, C., Piessens, J. and Goossens, H. (2005). Multiplex PCR For Simultaneous Detection Of Macrolide and Tetracycline Resistance Determinants In *Streptococci*. *Antimicrobial Agents and Chemotherapy*. **49**(11), 4798-4800.

Manani T. A., Collison E. K. and Mpuchane S. (2006). Microflora of minimally processed frozen vegetables sold in Botswana. *Journal of Food Protection*. **69**, 2581 – 2586.

McLauchlin, J., Mitchel, R.T., Smerdon, W. J. and Jewell, K. (2004). *Listeria monocytogenes* and listeriosis. A review of hazard characterization for use in microbial risk assessment of foods. *International Journal of Food Microbiology*. **92**, 15-33.

Mead, P. S., Slutsker, L., Dietz, V., McCraig, L. F., Breese, J. S., Shapiro, C., Griffin, P. M., and Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases* **5**, 607-623.

Miliotis, M. D. and Bier, J. W. (2003). International Handbook of Foodborne pathogens. Available from http://books.google.co.za.

Morris, A. K. and Masterton, R. G. (2002). Antibiotic resistance surveillance: action for international studies. *Journal of Antimicrobial Chemotherapy*. **49**, 7-10.

Morvan, A., Moubareck, C., Leclercq, A., Herve´-Bazin, M., Bremont, S., Lecuit, M., Courvalin, P. and Le Monnier, A. (2010). Antimicrobial Resistance of *Listeria monocytogenes* Strains Isolated from Humans in France. *Antimicrobial Agents and Chemotherapy*. **54**(6), 2728-2731.

Mosupye F. M. and Von Holy A. (2000). Microbiological hazard identification and exposure Microbiological hazard identification and exposure assessment of street food vending in Johannesburg, South Africa. *International Journal of Food Microbiology*. **61**, 137-145.

Murray, E. G. D., R. A. Webb and M. B. R. Swan. (1926). A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n. sp.). *J. Pathol. Bacteriol*. **29**, 407-439.

Nakamura, H., Hatanaka, M., Ochi, K., Nagao, M., Ogasawara, J., Hase, A. and Nishikawa, Y. (2004). *Listeria monocytogenes* isolated from cold-smoked fish products in Osaka City, Japan. *International journal of food microbiology*. **94**(3), 323-328.

Nicholson, F. A., Groves, S. J., and Chambers, B. J. (2005). Pathogen survival during livestock manure storage and following land application. *Bioresource technology*. **96**(2), 135-143.

Njagi, L. W., Mbathia P. G., Bebora L. C., Nyaga P. N., Minga U. and Olsen J. E. (2004). Carrier status for *Listeria monocytogenes* and other *Listeria* species in free range farm and market healthy indigenous chicken and ducks. *East African Medical Journal*. **81**, 529-533.

Nolan, D.A., Chamblin, D.C., and Troller, J.A. (1992). Minimal water activity levels for *Listeria monocytogenes* and *Listeria innocua*. *International Journal of Food Microbiology* **16**, 323-335.

Novais, C., Freitas, A. R., Silveira, E., Baquero, F., Peixe, L., Roberts, A. P., and Coque, T. M. (2012). Different genetic supports for the *tet (S)* gene in enterococci. *Antimicrobial agents and chemotherapy*. **56**(11), 6014-6018.

Nyenje, M. E., Tanih, M. F., Green, E. and Roland, N. (2012). Current Status of Antibiograms of *Listeria ivanovii* and *Enterobacter cloacae* Isolated from Ready-To-Eat Foods in Alice, South Africa., *International Journal of Environmental research and Public Health*. **9**, 3101-3114.

Oehme, F. W. (1973). Significance of chemical residues in United States food-producing animals. *Toxicology*. **1**, 205-215.

Oevermann, A., Di Palma, S., Doherr, M. G., Abril, C., Zurbriggen, A. and Vandevelde, M. (2010). Neuropathogenesis of naturally occurring encephalitis caused by *Listeria monocytogenes* in ruminants. *Brain Pathology*. **20**, 378-390.

Ojeniyi, B., Wegener, H. C., Jensen, N. E., and Bisgaard, M. (1996). *Listeria monocytogenes* in poultry and poultry products: epidemiological investigations in seven Danish abattoirs. *Journal of applied bacteriology*, **80**(4), 395-401.

Onyemelukwe, G. C., Lawande, R. V., Egler, L. J. and Mohammed, I. (1983). *Listeria monocytogenes* in Northern Nigeria. *Journal of Infections*. **6**, 141-145.

Ormerod, A. D., Reid, T. M. and Main, R. A. (1987). Penicillin in milk: its importance in urticaria. *Clinical Allergy*. **17**, 229-234.

Orsi, R. H. and Wiedmann, M. (2016). Characteristics and distribution of *Listeria spp*., including *Listeria* species newly described since 2009, *Applied Microbiology and Biotechnology*.

Osaili, T. M., Alaboudi, A. R., and Nesiar, E. A. (2011). Prevalence of *Listeria spp*. and antibiotic susceptibility of *Listeria monocytogenes* isolated from raw chicken and ready-to-eat chicken products in Jordan. *Food Control*, **22**(3), 586-590.

Panin, A. N., Violin, B. V. and Kovalev, V. F. (1997). Some problems due to antibiotic resistance and application of feed antibiotics in Russia, In: The Medical Impact of the Use of Antimicrobial in Food Animals, section 2.3.9. Geneva: World Health Organization.

Pesavento, G., Ducci, B., Nieri, D., Comodo, N., & Nostro, A. L. (2010). Prevalence and antibiotic susceptibility of *Listeria spp*. isolated from raw meat and retail foods. *Food Control*. **21**(5), 708-713.

Pérez-Trallero, E., Zigorraga, C., Artieda, J., Alkorta, M., and Marimón, J. M. (2014). Two outbreaks of *Listeria monocytogenes* infection, Northern Spain. *Emergence of Infecioust Disease*, **20**(12), 2155-2157.

Phillips, I. (1999). The use of bacitracin as a growth promoter in animals produces no risk to human health. *Journal of Antimicrobial Chemotherapy*. **44**, 725-728.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R. and Waddell, J. (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *Journal of Antimicrobial Chemotherapy*. **53**, 28–52.

Pirie. J. H. H. (1927). A new disease of veldt rodents, "tiger river disease". *Pub. South Africa. Inst. Med. Res*. **62**, 163-186.

Pizarro-Cerdá, J., Kühbacher, A., and Cossart, P. (2012). Entry of *Listeria monocytogenes* in mammalian epithelial cells: an updated view. *Cold Spring Harbor perspectives in medicine*, ***2***(11), 1-17.

Ponniah, J., Robin, T., Paie, M. S., Radu, S., Ghazali, F. M., Kqueen, C. Y., and Malakar, P. K. (2010). *Listeria monocytogenes* in raw salad vegetables sold at retail level in Malaysia. *Food Control*, **21**(5), 774-778.

Portnoy, D. A., Auerbuch, V. and Glomski, I. J. (2002)**.** The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *The Journal of Cell Biology*. **158**(3), 409-414.

Portnoy, D. A., Chakraborty, T., Goebel, W. and Cossart, P. (1992) Molecular determinants of *Listeria monocytogenes*pathogenesis. *Infection and Immunity*. **60**, 1263–1267.

Pourshaban, M., Ferrini, A. M., Mannoni, V., Oliva, B., & Aureli, P. (2002). Transferable tetracycline resistance *in Listeria monocytogenes* from food in Italy. *Journal of medical microbiology*, **51**(7), 564-597.

Poyart-Salmeron, C., Trieu-Cuot, P., Carlier, C., MacGowan, A., McLauchlin J., and Courvalin, P. (1992). Genetic basis of tetracycline resistance in clinical isolates of *Listeria monocytogenes*, *Antimicrobial Agents and Chemotherapy* **36**, 463–466.

Prescott, J. F. (1997). Antibiotics: miracle drugs or pig food?. *Canadian Veterinary Journal*. **38**, 763-766.

Prescott, J. F., Baggot, J. D. and Walker, R. D. (2000). Antimicrobial therapy in veterinary medicine 3rd edition. Wiley, 2000.

Ramaswamy, V., Cresence, V. M., Rejitha, J. S., Lekshmi, M. U., Dharsana, K. S., Prasad, S. P., & Vijila, H. M. (2007). *Listeria*-review of epidemiology and pathogenesis. *Journal of Microbiology Immunology and Infection*, **40**(1), 4.

Rorvik, L. M., Aase, B., Alvestad, T. and Caugant, D. A. (2003). Molecular epidemiological survey of *Listeria monocytogenes* in broilers and poultry products. *Journal of Applied Microbiology*, **94**, 633–640.

Rutter, J. M. (1997). Use of anti-infective drugs in the UK-types of use and conditions/legislation for application, In: The Medical Impact of the Use of Antimicrobials in Food Animals, Section 2.3.5. Geneva: World Health Organization.

Ryser, E. T. (1999) Food borne listeriosis. In *Listeria*, Listeriosis and Food Safety ed. Ryser, E. T. and Marth, E. H. 299–358. New York: Marcel Dekker.

Sarmah, A. K., Meyer, M. T. and Boxall, A. B. A. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere*. **65**, 725-759.

Schlech, W. F., and Acheson, D. (2000). Foodborne listeriosis. *Clinical Infectious Diseases*, **31**(3), 770-775.

Seeliger, H. P., and Hohne, K. (1979). Serotyping of *Listeria monocytogenes* and related species. *Methods Microbiology*. **13**, 31-49.

Seragusa, G. R. and Johnson, M. G. (1990). Monoclonal Antibody Specific for *Listeria monocytogenes*, *Listeria innocua* and *Listeria welshimeri*. *Journal of Applied and Environmental Microbiology*. **56**, 1897-1904.

Settepani, J. A. (1984). The hazard of using chloramphenicol in food animals. *Journal of the American Veterinary Medical Association*. **184**, 930-931.

Seveau, S. (2014). Multifaceted Activity of Listeriolysin O, the Cholesterol-Dependent Cytolysin of *Listeria monocytogenes. Subcell Biochemistry.* **80**, 161-195.

Sibelius, U., Schulz, E. C., Rose, F., Hattar, K., Jacobs, T., Weiss, S. and Grimminger, F. (1999). Role of *Listeria monocytogenes* exotoxins listeriolysin and phosphatidylinositol-specific

phospholipase C in activation of human neutrophils. *Infection and immunity*, **67**(3), 1125-1130.

Silvers, L. E. and Spires, C. D. (2002). Use of antimicrobial in hatcheries: A field assignment. FDA Veterinarian Newsletter, 17:1. www.fda.gov/AnimalVeterinary/NewsEvents/FDAVeterinarianNewsletter/ucm106872.htm. Date visited 04/09/2016.

Slutsker, L. and Schuchat, A. (1999). Listeriosis in humans. In: Ryser, E. T., and Marth, E. H. (Eds.), *Listeria*, Listeriosis and Food Safety, *2nd Edition*. Marcel Dekker, New york, pp. 75-96.

Smith, D. L., Harris, A. D., Johnson, J. A., Silbergeld, E. K. and Morris, J. G. (2002). Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proceedings of the National Academy of Sciences of the United States of America*. **99**, 6434-6439.

Smith, J. L., Drum, D. J. V., Dai, Y., Kim, J. M., Sanchez, S., Maurer, J. J., Hofacre, C. L. and Lee, M. D. (2007). Impact of antimicrobial usage on antimicrobial resistance in commensal *Escherichica coli* strains colonizing broiler chickens. *Applied and Environmental Microbiology*. **73**, 1404-1414.

Srinivasan, V., Nam, H. M., Nguyen, L. T., Tamilselvam, B., Murinda, S. E., and Oliver, S. P. (2005). Prevalence of antimicrobial resistance genes in *Listeria monocytogenes* isolated from dairy farms. *Foodbourne Pathogens & Disease*. **2**(3), 201-211.

Stockstad, E. L. R. (1950). Further observations on the animal protein factor. *Proceedings of the Society of Experimental Biology and Medicine*. **73**, 523-528.

Stolker, A. A. and Brinkman, U. A. (20050. Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals-a review. *Journal of Chromatography.A.* **1067**, 15-53.

Su, X., Zhang, J., Shi, W., Yang, X., Li, Y., Pan, H. and Meng, J. (2016). Molecular characterization and antimicrobial susceptibility of *Listeria monocytogenes* isolated from foods and humans. *Food Control*, **70**, 96-102.

Tappero, J. W., Schuchat, A., Deaver, K. A., Mascola, L., Wenger, J. D. (1995). Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts. *Journal of Clinical Infectious Diseases.* **273**, 1118-1122.

Tominaga, T. (2006). Rapid Descrimination of *Listeria monocytogenes* Strains by Microtemperature Gradient Gel Electrophoresis. *Journal of Clinical Microbiology* **44**, 2199-2206.

Tulkens, M., Mingeot-leclercq, M. P., and Glupczynski, Y. (1999). Aminoglycosides: activity and resistance. *Antimicrob. Agents Chemother*. **43**(4), 727.

Unnevehr, L. J. (2003). Food Safety in Food Security and Food Trade. International Food Policy Research Institute. http://www.ifpri.org/sites/default/files/publications/focus10.accessed September 2016.

USDA-FAS. 2007. Livestock and poultry: world market and trade. www.fas.usda.gov/psdonline

Usman, U. B., Kwaga, J. K. P., Kabir, J., Olonitola, O. S., Radu, S. and Bande1, F. (2016). Molecular Characterization and Phylogenetic Analysis of *Listeria monocytogenes* Isolated from Milk and Milk Products in Kaduna, Nigeria. *Canadian Journal of Infectious Diseases and Medical Microbiology*. **2016**, 1-7.

van Nierop, W. V., A. G. Duse, E., Marais, N., Aithma, N., Thothobolo, M., Kassel, R., Stewart, A., Potgieter, B., Fernandes, J., Galpin, S. and Bloomfield, S. F. (2005). Contamination of chicken carcasses in Gauteng, South Africa, by *Salmonella, Listeria monocytogenes* and *Campylobacter*. *International Journal of Food Microbiology*. **99**, 1–6.

Varaldo, P. E., Montanari, M. P. and Giovanetti, E. (20090. Genetic Elements Responsible for Erythromycin Resistance in *Streptococci*. *Antimicrobial Agents and Chemotherapy*. **53**(2), 343-353.

Wagner, M. and Allerberger, F. (2003). Characterization of *Listeria monocytogenes* recovered from 41 cases of sporadic cases in Austria by serotyping and pulsed-field gel electrophoresis. *FEMS Immunology and Medical Microbiology*. **35**, 227-234.

Walsh, D., Duffy, G., Sheridan, J. J., Blair, I. S. and McDowell, D. A. (2001). Antibiotic resistance among *Listeria*, including *Listeria monocytogenes*, in retail foods. *Journal of Applied Microbiology*. **90**, 517-522.

Wang, G., Qian, W., Zhang, X., Wang, H., Ye, K., Bai, Y. and Zhou, G. (2015). Prevalence, genetic diversity and antimicrobial resistance of *Listeria monocytogenes* isolated from ready-to-eat meat products in Nanjing, China. *Food Control*. **50**, 202-208.

Wang, X. M., Lü, X. F., Yin, L., Liu, H. F., Zhang, W. J., Si, W., Yu, S. Y., Shao, M. L. and Liu, S. G. (2013). Occurrence and antimicrobial susceptibility of *Listeria monocytogenes* isolates from retail raw foods. *Food Control*. **32**, 153-158.

Weller D, Andrus A, Wiedmann M. and Bakker, H. C. (2015). *Listeria booriae sp. nov*. and *Listeria newyorkensis sp. nov*., from food processing environments in the USA. *Inernational Journal of Systematic and Evolutionary Microbioly.* **65**, 286–292.

Wiedmann M., Barany F. and Batt C. A. (1993). Detection of *Listeria monocytogenes* with a Nonisotopic Polymerase ChainReaction-Coupled Ligase Chain Reaction Assay. *Journal of Applied and Enviromental Microbiology*. **59**, 2743-2745.

Wong, A. C. (1998). Biofilms in food processing environments. *Journal of Dairy Science*. **81**, 2765-2770.

Woodward, K. N. (1996). Side effects of veterinary drugs: postmarketing surveillance in the UK. *British Institute of Regulatory Affairs Journal*. **14**, 22-26.

World Health Organization. (2014). *Antimicrobial resistance: 2014 global report on surveillance*. World Health Organization.

Yan, H., Neogi, S. B., Mo, Z., Guan, W., Shen, Z., Zhang, S., Li, L., Yamasaki, S., Shi, L. and Zhong, N. (2010). Prevalence and characterization of antimicrobial resistance of foodborne *Listeria monocytogenes* isolates in Hebei province of Northern China, 2005–2007. *International Journal of Food Microbiology*. **144**(2), 310-316.

Zhang, J., Cao, G., Xu, X., Allard, M., Li, P., Brown, E. and Meng, J. (2016). Evolution and Diversity of *Listeria monocytogenes* from Clinical and Food Samples in Shanghai, China. *Frontiers in Microbiology*, **7**. 1138.

Zhang, W. and Knabel, S. J. (2005). Multiplex PCR assay simplifies serotyping and sequence typing of *Listeria monocytogenes* associated with human outbreaks. *Journal of Food Protection*. **68**, 1907-1910.

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