

**CONTAMINATION OF CHICKEN CARCASSES AND POULTRY PROCESSING
ENVIRONMENT WITH *LISTERIA* SPECIES IN ZARIA, NIGERIA**

BY

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ZARIA**

NOVEMBER, 2015

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ENVIRONMENT WITH *LISTERIA* SPECIES IN ZARIA, NIGERIA**

BY

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ZARIA, NIGERIA**

NOVEMBER, 2015

Declaration

I declare that the work in this dissertation entitled **Contamination of Chicken Carcasses and poultry Processing Environment with *Listeria* species in Zaria, Nigeria**” was performed by me in the Department of Veterinary Public Health and Preventive Medicine, under the supervision of Prof. J. U Umoh and J. Kabir

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at this or any other Institution.

Mohammed Salisu SADIQ

Signature

Date

Certification

This project dissertation entitled **CONTAMINATION OF CHICKEN CARCASSES AND POULTRY PROCESSING ENVIRONMENT WITH *LISTERIA* SPECIES IN ZARIA, NIGERIA** by Salisu Mohammed SADIQ meets the regulation governing the award of the degree of Master of Science in Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, and approved for its contribution to knowledge and literary presentation.

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Dedication

This Dissertation is dedicated to Almighty God for mercies, my parents Mr and Mrs Sadiq, Family and Friends for their support

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I would like to thank God, who is the head of my life. It is with his arms of protection and provision that I am able to make it from day to day. My heartfelt appreciation goes to my parents, Mr. and Mrs. Sadiq for all of the love, encouragement, financial support, prayers and guidance they have shown me and still showing me. It is because of their total support that I have the strength, resource and courage to pursue my educational and personal goals.

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Abstract

Listeria species are widely distributed in the environment and *L. monocytogenes* is the causative agent of listeriosis. This study was carried out to isolate and identify *Listeria* species from chicken carcasses and poultry processing environment, determine the antimicrobial susceptibility pattern of *Listeria* isolates to 12 commonly used antimicrobial agents and to determine haemolysin A (*hyl*) virulence gene carriage in *L. monocytogenes* isolates using polymerase chain reaction. A total of 412 samples were collected which comprises 308 from chicken carcasses (154 from broilers and 154 from local chickens) and 104 samples from poultry processing environment. From poultry processing environment, 26 swab samples were collected from surfaces of tables, floor, knives and bowls. *Listeria* species were isolated using pre-enrichment selective medium and *Listeria* species (spp) were identified by conventional biochemical tests and confirmed with Microgen ID-System. From the 412 samples, 40 (9.71%) were found to be contaminated with *Listeria* spp. Out of these 40 isolates, 2 (5%) were found to be *L. monocytogenes* while the remaining 38 isolates comprised *L. ivanovii* 32 (80%), *L. grayi* 4 (10%), and *L. welshimeri* 2 (5%) respectively. Distribution of the occurrence of *L. monocytogenes* by sample type and location showed that the 2 *Listeria monocytogenes* isolates were from broiler carcasses and one each from Sabo and Danmagaji markets respectively. Similarly, distribution of *Listeria* species in poultry processing environment showed that 15 (14.42%) *Listeria* species were isolated from poultry processing environment which are distributed as follows: knives 1 (3.85%), table 1 (3.85%), Floor 6 (23.08%) and 7 (26.92%). Antibiotic susceptibility testing showed that isolates were 100% resistant to amoxicillin,

tetracycline, vancomycin and erythromycin while the isolates had the least resistance to gentamicin (15%) and ciprofloxacin (30%). Antimicrobial profile of these isolates showed that they were all resistant to at least 5 antimicrobial agents. The 2 *Listeria monocytogenes* isolates (SBBC 116 and DBC 47) were subjected to PCR for the detection of virulence associated *hyl A* gene, the isolates were found to harbour *hyl A* gene (with an amplicon size of 702bp). Distribution *hyl A* by location and sample type showed that 1 of the two isolates (DBC 47) came from Danmagaji, while the other came from Sabo (SBBC 116) and both were found in broiler carcasses. This study therefore, showed that *Listeria* species contaminating chicken carcasses and poultry processing environment in Zaria, Nigeria are multi-drug resistant and showed virulence potential due to the presence of Listeriolysin A gene in *Listeria monocytogenes*. Consequently, good hygiene practices should be implemented when processing and handling chicken carcasses. Also, antibiotics usage in both human and veterinary medicine should be monitored and regulated in order to reduce the risk of increasing antimicrobial resistance.

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ABBREVIATIONS

| | |
|--------------------|---|
| α | Alpha |
| β | Beta |
| % | Percentage |
| μg | Microgram |
| $^{\circ}\text{C}$ | Degree Celsius |
| ABU | Ahmadu Bello University |
| ACA | Acriflavine –Ceftazidimine Agar |
| AFLP | Amplified fragment length polymorphism |
| AIDS | Acquired Immune Deficiency Syndrome |
| ATCC | American Type Culture Collection |
| A_w | Water activity |
| bp | Base Pair |
| CAC | Codex Alimentarius Commission |
| CAMP | Christine, Atkins, Munch-Petersen test |
| CDC | Centers for Disease Control and Prevention |
| DNA | Deoxyribonucleic acid |
| ERIC | Enterobacterial Repetitive Intergenic Sequences |
| FAO/WHO | Food and Agricultural Organization / World Health Organization of United Nations |
| FDA | Food and Drug Administration |

| | |
|-------------------|---|
| FSIS | Food Safety and Inspection Service |
| HACCP | Hazard Analysis and Critical Control Point |
| HIV | Human Immunodeficiency Virus |
| hr | Hour |
| <i>hly</i> A gene | Haemolysin A gene |
| ISO | International Organization for Standardization |
| LDE | <i>Listeria</i> Drug Efflux |
| LEB | <i>Listeria</i> Enrichment Broth |
| LGAs | Local Government Areas |
| LLO | Listeriolysin |
| LMP | Lithium chloride – Phenylethanol – Moxalactam Medium |
| LPM | Lithium Chloride – Phenylethanol Moxalactam |
| <i>mdr</i> L | Multidrug Efflux Transporter of <i>Listeria</i> |
| MEE | Multilocus enzyme electrophoresis |
| MHA | Muller-Hinton Agar |
| ML | Milliliters |
| MLST | Multilocus sequence typing |
| MMA | Modified MCBride Agar |
| Nacl | Sodium Chloride |
| PAHO | Pan American Health Organization |
| PALCAM | Polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin |
| PCR | Polymerase chain reaction |
| PFGE | Pulsed-Field Gel Electrophoresis |
| <i>pmr</i> A | Pneumoniae Multidrug resistance |

| | |
|----------------------|--|
| RAPD | Randomly Amplified Polymorphic DNA |
| REPs | Repetitive Extragenic Palindromes |
| rRNA | Ribosomal RNA |
| RTE | Ready to Eat |
| SPP | Species |
| SPSS | Statistical Package for Social Science |
| TSB | Tryptose Soy Broth |
| USDA-FSIS Service | United States Department of Agriculture – Food Safety and Inspection |

CHAPTER ONE

INTRODUCTION

1.1 Background

The genus *Listeria* is a Gram-positive, non-spore forming, rod-shaped bacteria of 0.4-0.5x0.5- 2 μm in size with rounded ends and can also be coccoid at times, occurring singly or in short chains and not encapsulated (Holt *et al.*, 2000). With the introduction of molecular methods, the diversity within the genus *Listeria* is better understood and now only contains *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, *L. grayi*, *L. marthii*, *L. rocourtiae*, *L. fleischmannii*, and *L. weihenstephanensis* (Zhang *et al.*, 2007, Halter *et al.*, 2013). *L. monocytogenes* and *L. ivanovii* are pathogenic species of humans and animals (Bhunia, 2008; Christelle, *et al.*, 2010).

Listeria species are Gram positive, facultatively anaerobic, motile, non-spore forming rod, catalase positive and they ferment sugars with production of acid without gas formation. *Listeria monocytogenes* is capable of causing serious invasive illness (listeriosis) in both humans and animals (Roberts and Greenwood, 2003). The organism grows over a wide temperature range from 1°C to 45°C, with an optimum around 37°C. *L. monocytogenes* can grow at pH values between 4.4 and 9.4, and at water activities ≥ 0.92 with sodium chloride (NaCl) as the solute (Dimic *et al.*, 2010).

Foodborne listeriosis, caused by *Listeria monocytogenes*, continues to be of major concern to the food industry and the general public because of its rate of lethality (at more than 25%) and its economic impact (McLauchlin *et al.*, 1991). Nearly 99% of human listeriosis has resulted from consumption of contaminated foods (Mead *et al.*, 1999). Listeriosis accounts for less than 1% of cases of foodborne illness, but for around 28% of the deaths

cases(Kumar, 2011). Listeriosis affects most often the pregnant women, foetus, elderly and immune compromised individuals (Swamina, 2006). *Listeria monocytogenes* is able to form biofilms especially if the nutrient conditions are quite favourable (Adriao *et al.*, 2008).

Listeria monocytogenes have been isolated from raw poultry in many countries including more industrialized ones, such as USA, UK, and Germany(Wilson, 1995; Rijpens,*et al.*, 1997; Tobia, *et al.*, 1997).Isolation of *L. monocytogenes* from chicken meat was reported previously by many investigators (Miettinen *et al.*, 2001; Akpolat *et al.*, 2004; Lekroengsin *et al.*, 2007; Keeratipibul and Lekroengsin, 2009; Ahmed and El-Atti, 2010).

In recent decades, antimicrobial resistance particularly multi-resistance has been considered as a public health problem worldwide(Van Den Bogaard and Stobberingh, 1999). The excessive and improper usage of antimicrobial agents could be responsible for the emergence of resistant bacteria. Although, the use of antimicrobials in poultry production phase has reduced the risk of infectious disease, it may lead to dissemination of antimicrobial-resistant bacteria including resistant strains of *Listeria* in the environment. The transmission of the resistant strains to humans via contaminated food products may have public health consequences (Filiouisis, *et al.*, 2009). It has been stated that the antimicrobial resistance of *Listeria* spp. is due to the acquisition of mobile genetic elements such as self-transferable and mobilizable plasmids; and conjugative transposons (Charpentier and Courvalin,1999).

1.2

Statement of Research Problem

Listeria species especially *L. monocytogenes* is of major concern to the meat industry and several outbreaks of listeriosis have in recent times occurred in Europe (Salvat *et al.*, 1995; Dorozynski 2000). Outbreaks of *L. monocytogenes* infections in Nigeria are rare because the disease produce febrile gastroenteritis in apparently healthy individuals and has a course that is similar to gastroenteritis caused by other organisms like *Salmonella* and *Campylobacter* spp. (FAO/WHO, 2004) which makes diagnosis more difficult.

The pathogen has been found regularly in poultry products and environments where meat is processed. It is capable of surviving in these environments for long periods of time (Salvat *et al.*, 1995). Frequencies of about 41–84% have been reported from broiler carcasses and raw chicken products (Lawrence and Gilmour, 1994; Franco *et al.*, 1995; Uyttendaele *et al.*, 1999). *Listeria monocytogenes* is able to grow and form biofilms on various food-processing surfaces, enhancing survival (Miettinen *et al.*, 2001). Improper cleaning and disinfecting of equipment in poultry houses and abattoirs may lead to contamination of the poultry carcasses and meat products.

As a result, *L. monocytogenes* has become an important organism of public health concern not just because of its zoonotic and economic importance but also because of the fact that it is ubiquitous in the environment and it is a major contaminant of water, milk, and milk products like cheese, vegetables, poultry, meat and Ready to Eat (RTE) foods (PAHO; 2001, CAC, 2009)

Listeriosis is a unique disease that represents a considerable public health concern because of its high mortality rate that reaches 20-40% in pregnant women, elderly, neonates and immunocompromised individuals (McLauchlin *et al.*, 2004; Liu, 2006; Wan Norhana *et al.*, 2010). Most cases of listeriosis appear to be foodborne, including those acquired during pregnancy. *L. monocytogenes* may cross-contaminate RTE meat and poultry products during post-processing steps such as slicing, peeling, and packaging (Murphy *et al.*, 2005). The yearly medical costs and productivity losses from the acute illness from foodborne *Listeria* in the USA are estimated to be twice the costs caused by *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli* O157:H7, respectively, despite the prevalence of these diseases being over the number of listeriosis cases, respectively (Fenlon, 1999).

Principally, listeriosis causes intra-uterine infection, meningitis and septicaemia. Listeriosis during pregnancy manifests as a severe systemic infection in the unborn or newly delivered infant as well as a mild influenza-like bacteraemic illness in the pregnant woman. Pregnancy and neonatal cases comprise 10% to 20% of the listeriosis cases (McLauchlin *et al.*, 2004). In adults and juveniles, the main presentations are as central nervous system infection and or septicemia. Most adult and juvenile cases occur amongst the immunosuppressed, e.g. patients receiving steroid or cytotoxic therapy or with malignant neoplasms (Buchholz *et al.*, 2001). Other groups include patients with Acquired immune deficiency syndrome (AIDS), diabetics, individuals with prosthetic heart valves or replacement joints and individuals with alcoholism or alcoholic liver disease. The incidence of infection increases with age, with the mean age of adult infection being over 55 years (McLauchlin *et al.*, 2004). Mild non-invasive listeriosis, with gastroenteritis and fever, has

also been reported in otherwise healthy individuals (Riedo *et al.*, 1994, Miettinen *et al.*, 1999, Aureli *et al.*, 2000). Veterinarian also stands the risk of becoming infected following handling of abortion materials from infected animals. This form of the disease is manifested as listerial dermatitis (Hirsh and Zee, 1999).

It is believed that occurrence of outbreaks is likely to have greater impact on the society later due to increasing number of susceptible population including elderly people and immunocompromised persons including those with HIV/AIDS as well as increasing consumption of ready to eat, refrigerated and extended shelf life foods (Pagotto *et al.*, 2006).

The first confirmed case of human listeriosis involving *L. monocytogenes* in northern Nigeria was reported by Onyemelukwe *et al.*, (1983) from patients with meningitis, meningoencephalitis, spontaneous peritonitis, arthritis, pelvic infection and urethritis.

1.3 Justification

Listeria monocytogenes is often associated with listeriosis and sometimes opportunistic infections in man and animals with a capacity to cause severe illness in pregnant women, neonates, in the elderly and immunosuppressed individuals(Acha and Szyfres, 2003).*L. ivanovii* is considered as a human and animal pathogen particularly in sheep (Selby *et al.*, 2006). Occasional reports of infection with *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, though generally avirulent, have been documented. The severity of listeriosis caused by *L. monocytogenes* in pregnant women and neonates and the economic loss associated with abortion of foetus in ruminant as a result of infection with *L. ivanovii* have necessitated the

need for a continuous surveillance for the presence of this disease in the population and the establishment of its level of occurrence (McLauchlin *et al.*, 2004).

In Nigeria, there are few records on the occurrence of *L.monocytogenes* in raw chicken carcasses and poultry processing environment. Though many researchers have worked and established various prevalence in food products like milk 5.3% (Yakubu *et al.*, 2012) , meat and meat products 4.4% (Ndahi *et al.*, 2013), coleslaw 4.7%, salad vegetables 8.5% and vegetable salad 6.2% (Ieren *et al.*, 2013) but there is dearth of information on *Listeria* in chicken carcasses and poultry processing environment. This gap in information on the prevalence of *L. monocytogenes* in chicken carcasses and poultry processing environment, has emphasized the need to carry out this study to generate baseline data and establish the prevalence of *Listeria* species especially *L. monocytogenes* in chicken carcasses and poultry processing environment in Zaria, Nigeria. With the spread of HIV/AIDS and prevailing immunocompromising diseases, the role of *Listeria* in the disease burden of Nigerians need to be elucidated. This is more required at the human-animal interface that occurs among Fulani and other rural animal rearers where public health education and biosecurity is low. In Nigeria, there is also a limited interest and curiosity within the medical profession on the role of pathogens like *Listeria* in diseases during pregnancy and infancy as indicated by the limited literature on the role of *Listeria* in the disease burden of Nigeria. The contribution of poultry, a widely reared and consumed, food animal will provide insights into the contribution of animal food sources in human infection in Nigeria.

1.4

Aim of the Study

The aim of this study was to determine the level of contamination and ascertain antimicrobial susceptibility patterns of virulent *Listeria* strains in chicken carcasses and poultry processing environment in Zaria, Nigeria.

1.5 Objectives of the Study

- i. To isolate and identify *Listeria* species in chicken carcasses and poultry processing environment.
- ii. To determine the antimicrobial resistance profile of *Listeria* species isolates to commonly used antibiotics.
- iii. To determine the virulence potential of *Listeria monocytogenes* isolates by detecting the *hlyA* gene.

1.6 Research Questions

- i. Are *Listeria* species contaminating chicken carcasses and processing environment in Zaria?
- ii. What are the antimicrobial resistant profiles of *L. species* isolates to commonly used antimicrobial agents?
- iii. Are *Listeria monocytogenes* isolates from chicken carcasses and processing environment harbouring *hlyA* gene?

CHAPTER TWO

LITERATURE REVIEW

2.1 Description of *Listeria* Species

The genus *Listeria* represents a group of closely related, Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria 0.5 μm in width and 1–1.5 μm in length, and with a low G+C content (Robinson *et al.*, 2000). *Listeria monocytogenes* is motile due to possession of one to five peritrichous flagella, which may be lost as the bacteria enter the host cell. Movement is still possible because the bacteria polymerize actin into long actin tails that propel the bacteria through the cytoplasm. Filaments, ranging in size of 6-20 μm , may develop in old cultures. Older cultures may also stain irregularly (Salyers and Whitt, 2002).

2.2 Virulence of *Listeria* Species

Reports on the virulence of *L. monocytogenes* had earlier been described (Racz 1969, 1972; MacDonald and Carter, 1980). *Listeria* infection is wide spread in animals and humans, involving a variety of visceral organs (Stelma *et al.*, 1987; Blood and Radostatis, 1990;

Bille and Doyle, 1991). Asymptomatic infections in both man and animals are common but clinical manifestations are only apparent when conditions such as stress and immunodepression are present (Blood and Radostatis, 1990; Schuchat *et al.*, 1991). The route of inoculation of *L. monocytogenes* leading to listeriosis is oral (Schlech *et al.*, 1983; Schuchat *et al.*, 1991; Farber and Peterkin, 1991). However, intra-peritoneal route has been reported as a means to studying listerial virulence in murine listeriosis using carrageenan an immunocompromising agent (Mielke *et al.*, 1993). The interrelationship of phenotypic and genetic virulence of *L. monocytogenes* is well documented. Phenotypically, the protein P60 - invasive associated protein (*iap*) is a major extracellular agent which plays a vital role in the *Listeria* invasion process and cell division (Kuhn and Goebel, 1989) in a comparative study of smooth *L. monocytogenes* and rough *L.monocytogenes* observed that the smooth produced more P60 than the mutants. The rough (mutants) produced reduced levels of P60, displayed a rough colony morphology as well as reduced adherence and invasiveness into the host cell types. Such mutants were reported to form long chains in which the organism is separated by double septa (Kuhn and Goebel, 1989).

The cell biology of the organism can be divided into four broad stages: internalization, escape from a vacuole, nucleation of actin filaments and cell to cell spread, especially in non-professional phagocytic cells. The gene coding for P60, (*iap*-gene) has been characterized to encode a protein of 484 amino acids with a significant sequence but contains no further hydrophobic sequences which might serve as membrane spanning domains (Bubert *et al.*, 1992).

However, virulence determinants of most pathogenic organisms (bacteria) are subject to environmental modulation and evidence suggests that similar strategies are employed by

L.monocytogenes and other *Listeria* species to optimize gene expression within the host (Mekalonas, 1992). Using Northern blot analysis technique, it was demonstrated that this thermoregulation is effected at the level of transcription with fewer transcripts corresponding to the virulence related genes: *hly*, *plcA*, *mpl* and *inlA* which are expressed at temperatures below 37°C (Leimeister–Wachter *et al.*, 1992). These mutants are unable to properly multiply and/or spread within infected host’s cells (Goebel *et al.*, 1993). However, under growth – limiting conditions (heat shock/stress or less nutrients) *prfA*- dependent proteins are preferentially synthesized which often indicate the existence of additional *prf A* regulated proteins in *L. monocytogenes* when subjected to such aforementioned conditions (Goebel *et al.*, 1993). However, the synthesis of catalase, superoxide dismutase enzymes and P60 which are all associated with the virulence of *L. monocytogenes* is not under the control of *Prf A* (Goebel *et al.*, 1993). The P60 related proteins found as major extracellular proteins in all *Listeria* species are not precursors to virulence, but only P60 of *L. monocytogenes* is able to potentiate virulence or restore the failure of R – mutants (exhibiting a drastically reduced synthesis of P60) to adhere to 3T6 mouse fibroblasts. Thus the P60 protein of *L. monocytogenes* differs characteristically from P60-related proteins of the non- virulent *Listeria* species (Goebel *et al.*, 1993).

2.3 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*. Immunoblotting performed

with affinity-purified antibody to listeriolysin 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 antisera to produce a hemolytic phenotype on blood agar plates (Leimeister- Wachter *et al.*, 1992). Southern blot 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 (Sibeliuss *et al.*, 1998). Other methods that can be employed to detect listeriolysin are; haemolysin assays and polyacrylamide gel electrophoresis, immuno-magnetic beads for listeria and *Listeria* exotoxin detection kits (Sibeliuss *et al.*, 1998).

2.4 Antimicrobial Resistance in *Listeria* Species

Listeria species 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). 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.

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, tetracycline 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 by 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).

2.5 Mechanisms of Antibiotic Resistance in *Listeria* Species

Antibiotic resistance in *Listeria* species is majorly due to acquisition of three types of movable genetic elements: self-transferable plasmids, mobilizable plasmids, and conjugative transposons (Charpentier and Courvalin, 1999).

Efflux pumps were reported to be associated with fluoroquinolone resistance in *Listeria* (Godreuil *et al.*, 2003). *Enterococci* and *Streptococci*, in particular, represent a reservoir of resistance genes for *L. monocytogenes*. The gastrointestinal tract of humans is considered the most probable site where the acquisition, by *Listeria* spp., of conjugative plasmids and transposons from *Enterococcus-Streptococcus* takes place (Doucet-Populaire *et al.*, 1991).

2.5.1 Antibiotic resistance mediation through conjugation

Conjugation is one of the important mechanisms used by *Listeria* to acquire antibiotic resistance. Several studies have described the conjugative transfer of plasmids and transposons carrying antibiotic resistance genes from *Enterococcus-Streptococcus* to *Listeria* and between *Listeria* species (Perez-Diaz *et al.*, 1982; Flamm *et al.*, 1984; Vicente *et al.*, 1988).

A broad-host-range plasmid, pIP510, first found in *Streptococcus agalactiae*, encodes resistance to chloramphenicol, macrolides, lincosamides and streptogramins. Plasmid pIP510 can be transferred by conjugation from *Streptococcus agalactiae* to *L. monocytogenes*, *L. murrayi*, and *L. grayi*. It replicates in *Listeria* to promote its own transfer between *Listeria* strains and back from *Listeria* to *Streptococcus* (Perez-Diaz *et al.*, 1982; Vicente *et al.*, 1988). Tn916, first discovered in *Enterococcus faecalis*, is a broad-host-range conjugative transposon, encoding resistance to tetracycline- minocycline.

According to Vicente *et al.*,(1988), Tn916 can be transferred by conjugation from *E. faecalis* to *L. innocua* at a frequency of 10^{-6} . Conjugative transfer of the Tn916-related transposon Tn1545, initially found in *S. pneumoniae*, was obtained from *E. faecalis* to *L. monocytogenes* *in vitro* and *in vivo* (Doucet-Populaire *et al.*, 1991; Poyart-Salmeron *et al.*, 1992). A broad-host-range plasmid of *E. faecalis*, *pAM β 1*, encoding resistance to erythromycin, was transferred to *L. monocytogenes* and back to *Enterococcus* (Flamm *et al.*, 1984). The conjugative transfer of a plasmid carrying the gene *vanA*, conferring resistance to glycol-peptide antibiotics, from *E. faecalis* to *L. monocytogenes*, *L. ivanovii* and *L. welshimeri* was also described (Biavasco *et al.*, 1996). Tetracycline resistance is believed the most frequent resistance trait in *L. monocytogenes* isolated from humans (Charpentier and Courvalin, 1999). Conjugative plasmids and transposons originating from *Enterococcus-Streptococcus*, are responsible for the emergence of resistance to tetracycline in *L. monocytogenes* clinical isolates (Poyart-Salmeron *et al.*, 1992). Plasmid pIP823, replicating by the rolling-circle (RC) mechanism, has a broad- host-range of both Gram-positive and Gram-negative bacteria. pIP823 was involved in high level trimethoprim resistance in an *L. monocytogenes* isolate from France (Charpentier *et al.*, 1995). Chloramphenicol resistance can be conjugatively transferred through plasmids in the pC223 family (Charpentier and Courvalin, 1999).

2.5.2 Active efflux of antibiotics

Antibiotic resistance mediated by efflux mechanisms was first reported in *L. monocytogenes* in 2000 (Mata *et al.*, 2000). The sequence of MdrL (multidrug efflux transporter of *Listeria*) protein is highly homological to the sequence of protein YfmO, a putative chromosomal multidrug efflux transporter of *Bacillus subtilis*. An allele-

substituted mutant of this gene in *L. monocytogenes* failed to pumpout ethidium bromide and presented increased susceptibility to macrolides, cefotaxime and heavy metals. Efflux pump Lde (*Listeria* drug efflux) is associated with fluoroquinolone resistance in clinical isolates of *L. monocytogenes* in France (Godreuil *et al.*, 2003). The Lde protein showed 44% homology with *PmrA* (pneumoniae multidrug resistance) of *Streptococcus pneumoniae*, which belongs to the major facilitator superfamily (MFS) of secondary multidrug transporters. The insertional inactivation of the gene (*ide*) results in increased susceptibility of fluoroquinolone in *L. monocytogenes* (Godreuil *et al.*, 2003).

2.6 Serotyping of *Listeria* Species

Many different subtyping methods have been used for the differentiation of *L. monocytogenes* such as conventional phenotypic methods and DNA-based subtyping methods. Conventional and phenotypic subtyping methods include serotyping, phage typing, and multilocus enzyme electrophoresis (Wiedmann, 2002). Serotyping is based on the antigens expressed on the bacterial cell surface, which are detected by antisera. Serotyping has been a classical tool in subtyping of *L. monocytogenes* and is based on somatic (O) and flagella (H) antigens. *L. monocytogenes* strains are divided into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979). However, over 95% of strains isolated from human cases and foods belong to serotypes 1/2a, 1/2b and 4b, therefore limiting the usefulness of serotyping in both epidemiological and contamination investigations (Farber and Peterkin, 1991).

2.7 Epidemiology and Distribution of *Listeria* Organisms

A variety of food products have been involved in most outbreaks , including soft cheese (Bille *et al.*,1990) and cooked meat products (Aguado *et al.*, 2004). These are considered of special risk due to the ability of *Listeria* to grow and survive in them. 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 (Morobeet *et al.*, 2009). The annual endemic disease rate varied from 2 to 15cases 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*, by Chintu and Bathirunathan (1975). 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 sero-status. 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 3 samples, *Neisseria meningitides* in 2, *Escherichia coli* in 1, *Listeria monocytogenes* in 2 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)(Ralovch *et al.*, 1984). 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 faeces 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 faeces 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).

L. monocytogenes can be found on poultry carcasses and in poultry processing plants. The prevalence of pathogens in chickens in many countries is well documented but their presence on 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, 1999; 2000). *Listeria* organisms are documented to be zoonotic; one of the

sources of infection is the domestic fowl where it could occur as inapparent infection. The carriage of *Listeria monocytogenes* and other *Listeria* in indigenous birds has not been documented in some African countries like Kenya (Njagi *et al.*, 2004).

The prevalence 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 faeces. 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 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).

2.8 Distribution of *Listeria* in Nigeria

The first case of human listeriosis in Nigeria was recorded in the northern part of Nigeria (Onyemelukwe *et al.*, 1983). Many researchers in Nigeria have carried out researches to establish prevalence for *Listeria* from many food and non-food sources. Oni *et al.*, (1989) detected *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 3a and 4b in pigs (36.1%), cattle (20.8%), dogs (20.0%), free range chicken (6.8%) and horses (68.4%) as well as sheep (13.0%) from Zango Abattoir, Zaria. Similarly, Akpavie and Ikheloa (1992) reported an outbreak of listeriosis among cattle in Ibadan, Nigeria. *Listeria monocytogenes* was reported by (Chukwu *et al.*, 2006a) in dry fish sold at Bukuru, K-vom and Vwang retail markets in Plateau state with the prevalence of 13.8%, 11.1%, 11.1% respectively. Also,

Chukwuet *et al.*, (2006b) reported listeriosis in African Buffalo with septicaemia and abortion in Plateau. Vegetables sold in Ado –Ekiti were also reported to be contaminated with *L. monocytogenes* at a prevalence of 73.75% (David and Odeyemi, 2007). A study carried in Sokoto on smoked fish showed a prevalence of 25% (Salihu *et al.*, 2008). A study of two anthropogenic lakes in Lokpa-Ukwu, Abia State, Nigeria by Nwachukwu *et al.*, (2010a) showed a prevalence rate of 91.67 and 79.17% for 24 samples analysed for each lake. *Listeria monocytogenes* was isolated from locally produced beverages sold at different markets in Abia state at a prevalence of 100% in five markets and above 60% in three markets (Nwachukwu *et al.*, 2009). Nwachukwu *et al.*, (2010b) also reported *L. monocytogenes* in *Telferia occidentalis*, *Pterocarpus soyauxii* and *Solanum macrocarpon* at a prevalence of 60%, 25% and 60% respectively. Ieren *et al.*, (2013), carried out a study to determine the occurrence of *Listeria monocytogenes* in processed and unprocessed salad and vegetables sold in Zaria, Nigeria. He obtained a prevalence of 4.7% for coleslaw, 4.4% for conventional vegetables, 8.5% for cabbage and 6.25% for lettuce. Daniel *et al.*, (2015) reported the contamination and antimicrobial susceptibility pattern of *Listeria* species in frozen and fresh chicken carcasses in three markets in Markudi, Nigeria. He reported prevalence of 6.67% in raw chicken carcasses and 13% in frozen chicken.

2.9 Listeriosis in Animals

Listeriosis has been detected in nearly all domestic animals (Gray and Killinger, 1966). Most listeriosis cases have been reported in sheep, among which *L. ivanovii* is also a significant cause of listeric infections, and also cows and goats, causing encephalitis, abortion, or septicemia (Beauregard and Malkin, 1971; Wilesmith and Gitter, 1986; Alexander *et al.*, 1992; Low and Donachie, 1997; Chand and Sadana, 1999; Wesley *et al.*, 2002). In sheep and cows, subclinical mastitis and gastroenteritis caused by *L.*

monocytogenes have also been reported (Jensen *et al.*, 1996; Clark *et al.*, 2004; Rawool *et al.*, 2007).

In monogastric animals, listeriosis is rare, and large epidemics with generalized listeriosis and acute deaths have been reported only in farmed chinchillas (Finley and Long, 1977; Wilkerson *et al.*, 1997; Wesley, 2007). In swine, the primary manifestation of listeriosis is septicemia, whereas in horses, abortions and encephalitis are also typical (Wesley, 2007). Listeriosis of fowls is probably secondary to viral infections, and typically causes septicemia with accompanying cardiac lesions (Cummins *et al.*, 1988; Wesley, 2007).

Pathogenesis of *Listeria* spp. in fish differs from that in other animals. *L. monocytogenes* is less pathogenic to fish than to mice, whereas *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welchimeri*, which have little or no virulence in mammals, are virulent in fish (Menudier *et al.*, 1996).

In livestock, listeriosis is associated with indoor housing and consumption of bad quality feed, especially silage (Wilesmith and Gitter, 1986; Wiedmann *et al.*, 1996; Wesley, 2007). The sensitivity of pregnant animals to listeriosis has led to epidemics in which the only symptoms were abortions (Wilesmith and Gitter, 1986).

Symptomless faecal carriage of *L. monocytogenes* has been reported in primates, other mammals, and birds (Husu, 1990; Iida *et al.*, 1991; Miettinen and Wirtanen, 2006; Ho *et al.*, 2007; Lyautey *et al.*, 2007; Hellström *et al.*, 2008; Esteban *et al.*, 2009). The highest prevalence, up to 30%, has been reported in cattle followed by other ruminants, whereas companion animals seldom carry this species (Embil *et al.*, 1984; Husu, 1990; Iida *et al.*, 1991; Lyautey *et al.*, 2007; Esteban *et al.*, 2009).

2.10 *L. monocytogenes* in Food-Processing Environments

In addition to raw and processed foods, equipment, contact and noncontact surfaces, and protective clothing of employees in various food-processing plants have been proven to be contaminated with *L. monocytogenes* (Autio *et al.*, 1999; Giovannacci *et al.*, 1999; Miettinen *et al.*, 1999a; Chasseignaux *et al.*, 2001; Keto-Timonen *et al.*, 2007; Berzins *et al.*, 2010). The most heavily contaminated objects have been complex equipment in contact with large product surfaces, such as coolers, conveyers, and cutting, slicing, and brining machines that are often difficult to clean, so they can maintain the contamination in factories despite regular cleaning and disinfecting (Autio *et al.*, 1999; Miettinen *et al.*, 1999a; Lundén *et al.*, 2003b; Keto-Timonen *et al.*, 2007; Berzins *et al.*, 2010).

L. monocytogenes may persist in food-processing plant for years (Rørvik *et al.*, 1995; Miettinen *et al.*, 1999a; Keto-Timonen *et al.*, 2007). Persistent contamination is typically caused by a few strains recurrently isolated from the same or changing sampling sites, in the presence of a background of several other sporadically occurring strains (Giovannacci *et al.*, 1999; Miettinen *et al.*, 1999a; Lundén *et al.*, 2003b; Keto-Timonen *et al.*, 2007; Berzins *et al.*, 2010). Persistent strains have been reported to tolerate particular disinfectants better than sporadic strains (Aase *et al.*, 2000; Lundén *et al.*, 2003a), possibly because the persistent strains attach more firmly to surfaces that contact food (Norwood and Gilmour, 1999; Lundén *et al.*, 2000; Latorre *et al.*, 2011). Attached cells tolerate mechanical and chemical cleaning and disinfecting better than free-living cells (Norwood and Gilmour, 2000; Mah and O'Toole, 2001; Renier *et al.*, 2011). The adherence of *L. monocytogenes* enables the formation of a biofilm, a surface-attached microbial community in which the resistance and persistence of the organism in food production lines is increased (O'Toole *et al.*, 2000; Renier *et al.*, 2011). The biofilm-forming ability of strains varies, with some strains being able to form three-dimensional mushroom-shaped biofilms,

while others produce sparse aggregates or monolayers (Chae and Schraft, 2000; Kalmokoff *et al.*, 2001; Borucki *et al.*, 2003). Surface material, environmental condition, and the presence of other bacteria affect the survival of *L. monocytogenes* in a biofilm (Bremer *et al.*, 2001; Midelet and Carpentier, 2002; Nilsson *et al.*, 2011; Bae *et al.*, 2012b; Kostaki *et al.*, 2012). Contamination may enter the processing plant from multiple sources, and the main source has been suspected to be raw material (Lawrence and Gilmour, 1995; Ojeniyi *et al.*, 1996; Giovannacci *et al.*, 1999; Katzav *et al.*, 2006; Berzins *et al.*, 2010; Hellström *et al.*, 2010). Several studies, however, have shown that raw material was free of *L. monocytogenes*, or that different *L. monocytogenes* strains were found in the raw materials and in the processing environment or processed foods (Rørvik *et al.*, 1995; Autio *et al.*, 1999; Hoffman *et al.*, 2003). Other possible contamination sources could be personnel, pests, insects, and soil (Iida *et al.*, 1991; El-Shenawy, 1998; Sauders *et al.*, 2005; Schoder and Wagner, 2011; Pava-Ripoll *et al.*, 2012). Persistent contamination has been attributed to the transfer of processing machinery between food processing plants (Lundén *et al.*, 2002).

2.11 *L. monocytogenes* in Foods

The prevalence of *L. monocytogenes* in carcasses and raw foods varies greatly. The highest prevalence have been reported in poultry carcasses and poultry meat, whereas in most studies it is relatively seldom found in beef and pork carcasses, raw milk, and fresh produce. The most commonly contaminated RTE foods of animal origin have been reported to be fermented, air-dried, or cold-smoked meat and fish products that have not undergone heat treatment that reduces the occurrence of the bacterium (Markkula, 2013).

The prevalence of *L. monocytogenes* in foods increases during processing (Eklund *et al.*, 1995; Lawrence and Gilmour, 1995; Rørvik *et al.*, 1995; Autio *et al.*, 1999; Berzins *et al.*, 2010). Several studies have shown that the main strains contaminating the final products

originate from the processing environments and are different from the strains present in raw material (Lawrence and Gilmour, 1995; Rørvik *et al.*, 1995; Autio *et al.*, 1999; Giovannacci *et al.*, 1999). Brining by injection has been associated with *L. monocytogenes* contamination of cold-smoked pork (Berzins *et al.*, 2007). Complex processing machines in contact with large product surfaces, including cutting, brining, and slicing machines, efficiently spread the contamination to the many products of a particular plant (Autio *et al.*, 1999; Berzins *et al.*, 2010). When the process does not include heat treatment, raw material may also serve as a direct source of contamination of the final products (Keto-Timonen *et al.*, 2007).

2.12 Isolation and Identification of *Listeria* Species

The media utilized in the isolation of *L. monocytogenes* and other *Listeria* species are selective and differential. They could be either liquid or solid. Many of these media contain antibiotics to which *Listeria* species are resistant (Klinger, 1988). However, the isolation of the organisms from non-sterile samples like soil, water, faeces, food and other environmental sources rely mostly on cultural procedures. These procedures are cold enrichment in non-selective and selective enrichment broths followed by plating on selective agar plates. Nevertheless, the organisms are often isolated from blood, cerebrospinal fluid, amniotic fluid, placental tissue and foetal tissue biopsy materials. These latter specimens can be directly cultured on agar containing 5% sheep, horse or rabbit blood and Nalidixic acid, Palcam agar and Oxford *Listeria* selective agar (Schuchat *et al.*, 1992; Swaminathan and Feng, 1994).

Other agents readily utilized to enhance the isolation of *L. monocytogenes* include aesculin (Seeliger and Jones, 1986). This is a glycoside found in the leaf and bark of horse chestnut

trees. Hydrolysis of aesculin or salicin, a glycoside found in willow branches, releases the glucose for metabolism and leaves the aglucone. This gives rise to O – diphermols or catechols. Catechols can form co-ordinate compounds with transition metals such as iron. This reaction is used by many bacteria in the formation of iron – binding siderophores. In the case of *Listeria* species, the released catechol is utilized as an indicator of aesculin hydrolysis to aesculitine forming dark precipitates with the iron salts in the medium (Curtis *et al.*, 1989).

Cold enrichment at 4°C in non- selective broth medium has been utilized for the isolation of the *Listeria* species since Gray *et al.*, (1948) reported the psychrotropic characteristics of *L. monocytogenes*. In view of the disadvantages of cold enrichment in non-selective broth, enriched medium containing some inhibitory chemicals and antibiotics have been developed for the isolation of *Listeria* species from specimens (Klinger, 1988; Schuchat *et al.*, 1991; Swaminathan and Feng, 1994). The reagents mostly used in liquid enrichment media to render them selective for *Listeria* species include nalidixic acid, lithium chloride, acriflavine, glycine, anhydride, nitrofurazone, potassium thiocyanate and potassium tellurite (Klinger, 1988). However, in recent times, the commonly used selective enrichment broths are: (a) United States Food and Drug Administration Medium (USFDA) – prepared from tryptic soy broth, acriflavine and nalidixic acid (Lovett, *et al.*, 1987; Lovett, 1988), (b) United States Department of Agriculture – Food Safety and Inspection Service (USDA – FSIS) broth – containing aesculin, acriflavine and nalidixic acid, (c) Oxford *Listeria* selective agar and (d) Palcam broth agar (Curtis *et al.*, 1989; Van-Netten *et al.*, 1989). McBride and Girrad (1960) developed the first selective plating agar medium for the cultivation of *L. monocytogenes* and other *Listeria* species. Their formulation contained blood, phenylethanol agar-base, lithium chloride and glycine. However, this medium was

modified by the removal of the blood component. This modification also favoured isolation of *L. monocytogenes* from foods (Lovett, 1988).

In addition to the above, Lee and McClain (1986) has also improved the selectivity of McBride medium by increasing the concentration of the constituents and substituted glycine anhydride, moxalactam, for glycine. They called the improved, medium Lithium chloride – phenylethanol – moxalactam (LPM) agar. This is highly selective for the isolation of *L. monocytogenes*, even from heavily contaminated specimens. Nevertheless, neither the former or improved McBride had any *Listeria* differential property except by examination using Henry's illumination which gives blue-grey colouration on *Listeria* organisms.

In order to overcome the disadvantages of the earlier formulated growth media, some workers developed differential media for presumptive identification of *Listeria* species on solid culture media. Thus Curtis *et al.*, (1989) and Van-Natten *et al.*, (1989) developed separately two differential *Listeria* media (Oxford *Listeria* agar and Palcam agar), by incorporating differential agents into the media. On the Palcam agar, *Listeria* colonies appear grey-green, approximately 2mm in diameter and have black – sunken centers with a black – halo against cherry red background (Van-Natten *et al.*, 1989). *L. monocytogenes* and other *Listeria* species colonies appear black on Oxford agar having 1mm – 3mm in diameter after 24-48 hours and are surrounded by black halo against grey background of the medium (Curtis *et al.*, 1989).

Several investigations have compared the various selective broth as well as solid agar media for the isolation and identification of *Listeria* species as a result of observations made by a number of workers in this field. These observations include the enhancement of

selectivity of enrichment and isolation media, improvement of the ease of detection of low numbers of *Listeria* species in samples, the shortening of culture time period and detection of heat injured or stressed organisms that may rejuvenate on cold storage of finished products.

The classical approach to the identification of bacteria is cultural. This approach involves subjecting suspected samples to a series of tests designed to isolate and identify micro-organism possessing a profile of designation of *Listeria* species at the strain and species levels, respectively.

The classic method for the isolation of *L. monocytogenes* and other *Listeria* species from biological samples is often by cold enrichment at 4°C for several weeks in a suitable medium (Gray *et al.*, 1948; Rodriguwez *et al.*, 1984). However, due to the long incubation period which is not productive in emergency cases, emphasis has been placed on effective direct enrichment. Buchanan (1990) suggested the modification of University of Vermont *Listeria* enrichment broth. In this broth, after 24 hours of sample incubation, there will be black precipitates showing aesculin utilization by the suspect organism. They, however, observed that this procedure yielded more false negatives when applied in the analysis of a large number of dairy and environmental samples.

A good number of enrichment broth procedures have been composed and some suffer from poor design and very low positive rates. Pini and Gilbert (1988) composed the cold enrichment of USFDA procedure and reported that the procedure was more effective for the isolation of *L. monocytogenes* from chicken and cheese than ordinary cold enrichment. Contrary to Pini and Gilbert (1988) report, Doyle and Schoeni (1987) had earlier reported that cold enrichment was superior to USFDA method for the isolation of *L. monocytogenes*

from soft cheese. In another study, Farber and Peterkin (1991) however, observed that FDA method was better than cold enrichment as earlier reported by Pini and Gilbert (1988) for the isolation of *L. monocytogenes* from artificially contaminated single-strength orange juice. In a separate study, (Lammerding and Doyle 1989; Curtis *et al.*, 1989) carried out parallel evaluation of FDA, USDA-FSIS and University of Vermont modified *Listeria*-selective broth and cold enrichment procedures for the recovery of *L. monocytogenes* from dairy products. From the outcome of their separate investigations, they concluded that the University of Vermont(UVM) procedure was best followed by USDA – FSIS, FDA and then the ordinary cold enrichment.

Hayes *et al.*, (1990) compared cold enrichment and USDA-FSIS for the isolation of *L.monocytogenes* and concluded that USDA procedure was significantly better ($P < 0.001$) than ordinary cold enrichment. They observed efficiencies of 96% and 59% in the two methods respectively. In the recent past, Swaminathan and Feng (1994) observed that no matter the broth, if the ideal antibiotic and reagents are incorporated, the broth would support the growth of *L.monocytogenes* and other species especially when incubated in cold conditions. Growth is best at 30°C- 37°C as well as 4°C with a minor change in their fatty acid composition (Puttmann *et al.*, 1993).After the number of *L. monocytogenes* has been increased by enrichment broth steps, the next phase of microbiological analysis is isolation of the desired pathogen. This is achieved through the use of one or more selective or differential plating media. In the case of *L.monocytogenes*, a number of plating methods have been developed with varying degrees of success. However, McBride *Listeria* agar has served as the basis for a number of other *Listeria* agar formulations. Examples of isolation media for *Listeria* include Centre for disease Control (CDC) modified McBride *Listeria* agar (MLA), Acriflavine-Ceftazidimine agar (ACA), FDA modified McBride agar (MM),

Lithium chloride-phenylethanol moxalactam (LPM) agar, Oxford *Listeria* selective agar and Palcam *Listeria* agar (Jones, 1989; Curtis *et al.*, 1989; Van-Natten *et al.*, 1989).

2.13 Confirmatory Tests for *Listeria monocytogenes*

The final phase of the analysis for *L. monocytogenes* is the confirmation of the genus identification and subsequent speciation of positive *Listeria* isolates. To confirm the genus, the following parameters have been internationally accepted. These include aesculin hydrolysis on Oxford *Listeria* agar, or Black halo-formation on Palcam agar and β -haemolysis on sheep blood agar (Curtis *et al.*, 1989; Van Netten *et al.*, 1989). Others are Gram-positive coccoid to short rods, tumbling motility, catalase positivity, oxidase-negative, Voges-Proskauer production from glucose (Buchamann, 1990). Other suggested tests are utilization of Xylose, Mannitol, Rhamnose and CAMP test using *Staphylococcus aureus* and *Rhodococcus equi* and the pathogenicity respectively (Buchanan, 1990).

2.14 Molecular Subtyping Technique

2.14.1 Multilocus sequence typing (MLST)

The MLST technique was developed by Maiden *et al.*, (1998) and it based on the principles of MLEE. This technique targets multiple genetic loci among different strains within a species that have changed slowly over time. MLST uses automated DNA sequencing to characterise the alleles present at different housekeeping genes to subsequently define an allelic profile or sequence type for each isolate (Salcedo *et al.*, 2003). The advantage of this technique is that the results, the sequence data, are unambiguous. These results can be

placed in a central database and it can be accessed through a web server, thus enabling exchange of molecular typing data for global epidemiology (Maiden *et al.*, 1998).

2.14.2 PCR-based subtyping techniques

PCR has many advantages including high input processing, a level of automation, and a relatively short reaction time. It also shows an increase in sensitivity over culturing methods. The inability of some cells to grow on selective media due to low numbers is also excluded as PCR amplifies the specific genetic signals from as little as only a few cells (Shearer *et al.*, 2001; Aznar and Alarcón, 2002). Arbitrarily primed PCR (AP-PCR) and randomly amplified polymorphic DNA (RAPD) Both RAPD and AP-PCR uses low-stringency PCR amplification to generate anonymous DNA fragments that are strain-specific. The primers used are single, short (approximately 10 bases long for AP-PCR and 10 – 15 bases for RAPD) and of arbitrary sequence. These primers are used at relatively low temperatures (around 36 °C) during PCR, thus effectively lowering the stringency of the primer-annealing temperature (Welsh *et al.*, 1990). This in turn allowing the annealing of a random primer that shows a perfect match of two or three nucleotides between the template strand and the 3' end of the primer. This three nucleotide sequence can presumptively be found in each 64 nucleotide sequences (43 permutations). When two such events (annealing and priming) occur within a certain distance from one another, the sequence that lies between these two sites can be amplified (Farber and Addison, 1994; O'Donoghue *et al.*, 1995). A comparison between strains or isolates can only be made if the same primer is used for all the test samples. Otherwise, the fragments yielded would be of no importance as the amount and sizes of the fragments would conflict because of the different primers used. The test samples also have to be pure cultures to avoid

contamination and subsequent deceiving or misleading results (Lawrence and Gilmour, 1995). This technique has been used to trace the source of *L. monocytogenes* contamination in both poultry (Lawrence and Gilmour, 1995) and vegetable (Aguado *et al.*, 2004) processing plants.

2.14.3 Amplified fragment length polymorphism (AFLP)

AFLP is a fingerprinting technique based on the amplification of genomic restriction fragments to create a restriction pattern of the amplified bands (Aarts *et al.*, 1999). DNA is digested with two restriction enzymes, one that cuts infrequently and another that cuts more frequently (Blears *et al.*, 1998). After the DNA is digested, it is ligated with adapter oligonucleotides (Aarts *et al.*, 1999). It is then amplified by PCR using adapter-specific primers (Keto-Timonen *et al.*, 2003). This will result in the amplification of only those fragments that have the same complementary sequence between the adapter and the restriction site and will thus result in amplification of 1/16 of the bands (Aarts *et al.*, 1999). After PCR, the products are separated by polyacrylamide gel electrophoresis, to yield highly informative, polymorphic patterns (Ripabelli *et al.*, 2000; Guerra *et al.*, 2002; Keto-Timonen *et al.*, 2003).

AFLP is a sensitive, reproducible and highly discriminatory method that can be used to discriminate between species of the genus *Listeria*, as well as between different *L. monocytogenes* strains (Ripabelli *et al.*, 2000; Guerra *et al.*, 2002; Keto-Timonen *et al.*, 2003). The disadvantage of AFLP is the ligation of adapters, which is not only a time-consuming step, but also adds ambiguity to the method (Liu, 2006).

2.14.4 REP-PCR

Listeria monocytogenes, like other prokaryotic organisms, contains a genome with repetitive sequence elements, which are randomly dispersed throughout the genome. They include Enterobacterial Repetitive Intergenic Consensus Sequences (ERICs) which contains 124 – 147 base pairs (bp) and have a highly conserved central inverted repeat and repetitive extragenic palindromes (REPs) which contain 35 – 40 bp and which have an inverted repeat. These ERIC and REP sequences are both ideal primer binding sites for PCR amplification and could be used for both species and strain discrimination.

2.15 Transmission of Listeriosis

Listeriosis is primarily a foodborne infection. Ingestion of contaminated food products has been identified as the source of infection in both sporadic and outbreak-associated cases. Direct contact transmission of *Listeria* species from animals to humans and has been documented in veterinarians, farmers and abattoir workers. Vertical transmission from mother to neonate occurs transplacentally or through an infected birth canal. The approximate fatality rate is 30% that may increase up to 75% in high risk groups, such as pregnant women, neonates and immune compromised adults (Jalali and Abedi, 2007; Mead *et al.*, 1999; Low and Donachie, 1997).

2.16 Contamination of Chicken Carcasses and Poultry Processing Environment

Listeria species are widely spread in nature, being commonly found in food products and food processing environment as a biofilm which has the capacity to multiply at refrigeration temperatures (Muriama *et al.*, 1996). *Listeria monocytogenes* is the most

common bacterial contaminant on chicken carcasses at slaughterhouses (Lindblad *et al.*, 2006). Chicken can be contaminated with *L. monocytogenes* during production, in the processing plant, and in storage, and chicken products can become contaminated through contaminated raw material or cross-contamination during preparation, cooking, and serving food (Mylius *et al.*, 2007; Osailiet *al.*, 2011).

CHAPTER THREE

MATERIALS AND METHOD

3.1

Materials

3.1.1 Culture media

Media were obtained from Oxoid (Basingstoke, England) and they comprise *Listeria* Enrichment Broth, *Listeria* Selective agar, Nutrient agar, Tryptone Soya agar, Blood agar

base, Muller- Hinton agar, Andra Peptone Water, Buffered peptone water and Sodium Chloride.

3.1.2 Glassware

Glass and disposable Petri dishes, Microscopic Glass slide, Measuring cylinder, conical flask, Universal bottles, Bijou bottles, Test tubes, Pipettes.

3.1.3 Antibiotic discs and their concentrations

Chloramphenicol (30µg), Gentamycin (10µg), Amoxicillin (10 µg), Ciprofloxacin (5µg), Streptomycin(10µg), Tetracycline (30µg), Vancomycin (30µg), Sulfamethoxazole-Trimethoprim (25µg) Erythromycin(15µg), Trimethoprim (5µg), Cefoxitin (30 µg), Nitrofurantoin (50µg) (Oxoid, Basingstoke, England).

3.1.4 Reagents or chemicals

Crystal violet, Lugol iodine, Safranin dye, Hydrogen peroxide, acetone, Sanitizer, Detergents, Acetone- alcohol, phenol, chloroform ethidium bromide, phosphate saline buffer and isoamylalcohol, MgCl₂, Agarose gel, and dNTP.

3.1.5 Rapid kits

Microgen™ *Listeria* - ID System (Microgen bioproducts)

3.1.6 Molecular apparatus

Primers and enzyme

Taq polymerase, primers for *hlyA* gene (LM1: 5'-CCTAAGACGCCAATCGAA -3' (Forward) and LM2: 5'- AAGCGCTTGCAACTGCTC -3' (Reverse) (Lakicevic *et al.*, 2010).

3.1.7 Laboratory equipment

Incubator, Refrigerator, Stomacher laboratory blender (SWARD, London) , Weighing balance, Hand gloves, Racks, Autoclave, Cotton wool, Sterile Swab stick, Ice-man cooler, Masking tape, Markers, Aluminum Foil Paper, Bunsen Burner, Wire loop and pin, Needle and syringe, Antibiotic disc dispenser , Pasteur pipettes, Hot air oven, Water bath, DNA thermal cycler (MJ Research Inc.).

3.1.8 Supplements

Listeria Enrichment Supplement, *Listeria* Selective Supplements (Oxoid, Basingstoke, England)

3.1.9 Sugars

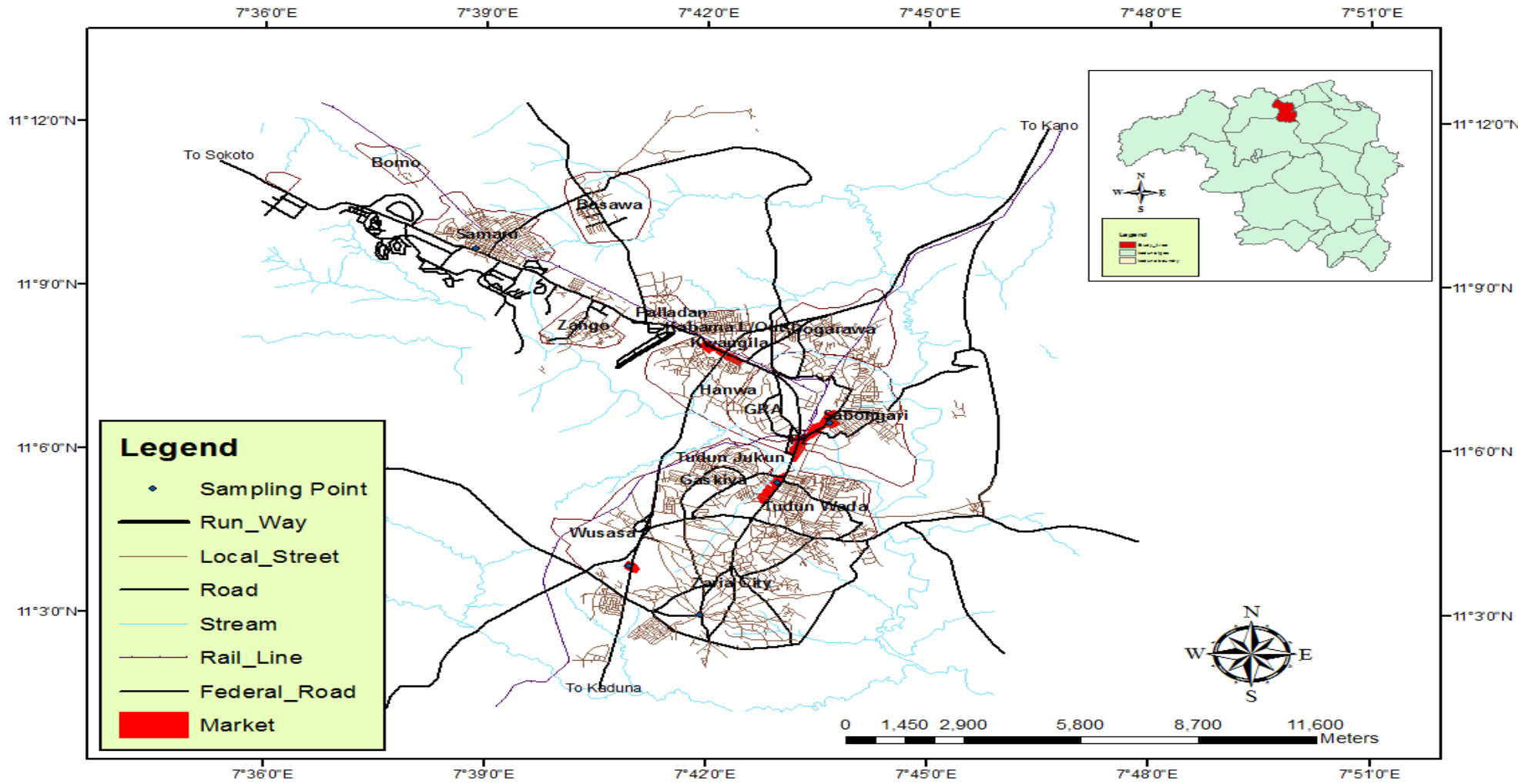
Rhamnose, Xylose, Mannitol(Oxoid, Basingstoke, England).

3.2

Study Area

This study was carried out in Zaria, Nigeria. Zaria lies between latitude $11^{\circ} 7'$, $11^{\circ} 12'$ N and longitudes $7^{\circ} 41'$ E. Zaria covers a land mass of 146,016 sq. kilometres approximately (Mamman, *et al.*, 2000) and with an estimated population of 547,000 and a growth rate of 3.5 per annum. Zaria is divided administratively into Zaria and Sabon Gari LGAs (Ministry of Economic Development, 1996).

Agricultural activity in Zaria can be divided into two (2) types: rained- fed (from May to October) and irrigation farming in the dry season (from November to April). It is characterized by tropical climate, a monthly mean temperature ranging from 13.8 to 36.7⁰ C and an annual rainfall of 1092.8mm (Agbogu *et al.*, 2006).



Source: Google Earth Pro 4.0, 2014

A cross sectional study was carried out to determine contamination of chicken carcasses and poultry processing environment with *Listeria* species. List of live bird markets were used as the sampling frame with the markets being the sampling units. Samples that were collected included representative portions of chicken carcasses, swab from working tables/slabs during or after slaughter, buckets/bowls, knives and floor. Four markets in Zaria were randomly selected for this study and they include Samaru, Tudunwada, Sadon gari, and Danmagaji markets.

3.4 Determination of Sample Size

A total of 412 chicken carcasses and swabs from poultry processing environment were collected and investigated for *Listeria* species contamination during the period of August-December 2014. Sample size was calculated using the equation outlined by Thrusfield (1997)

3.4.1. Sample size determination of chicken carcasses

$$n = \frac{Z^2 Pq}{d^2}$$

Where n = sample size

P = Prevalence (27%)

q = 1-P

d² = allowable error (5%)

By taking 27% prevalence rate of *Listeria* species in raw chicken (from a pilot study).

Where:

$$n = \frac{1.96^2 \times 0.115 (1-0.115)}{0.05^2} = 303$$

= 303 samples

3.4.2 Sample size of poultry processing environment

One hundred and four samples were collected from poultry processing environment in Zaria, Nigeria based on sample availability. However, a total of 412 samples were collected from chicken carcasses and poultry processing environment which comprises 308 chicken carcasses and 104 samples from poultry processing environment (samples were collected based on availability).

3.5 Sample Collection and Analysis

3.5.1 Sample collection

A total of 412 samples were collected from raw chicken carcasses (308) and poultry processing environment (104) using convenient sampling technique from four markets in Zaria. Swab samples were taken from food contact surfaces and non – food contact surfaces (floor and buckets/bowls) with sterile stick swab (wipe sampling device).

3.5.1.1 Chicken carcasses samples

From the 308 chicken carcasses collected, 154 were from broiler carcasses and 154 from local chicken carcasses. 77 chicken carcasses were collected from each market (39 broiler carcasses and 38 local chicken carcasses). From the raw dressed carcasses, about 12 grams of samples were taken from the neck skin of the chicken carcasses using a sterile scapel and transferred aseptically into a well labelled sterile transparent nylon bags.

3.5.1.2 Poultry processing environment samples

The 104 Samples collected from poultry processing environment comprises 26 samples from table working surfaces, 26 samples from knife surfaces, 26 samples from bowl and 26 samples from the floor. For the tables and floors, their surfaces were divided into quadrants. Three quadrants were randomly selected for sampling. Within the quadrants, an area of 10cm³ swabbed and the samples were pooled while smaller surfaces like knives and bowls; about 75% of the total surface were covered. The swabs were immediately and aseptically transferred in to sterile universal bottles containing normal saline and were shaken until the mixture became homogenous.

3.5.2 Sample handling and transportation

Samples were packed, labeled appropriately and transported in a Coleman box containing ice packs (to maintain the samples between 1°C and 8°C) to the Bacterial Zoonoses Laboratory in the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria, Nigeria for analysis. Samples were processed within six hours of collection

3.5.3 Cultural isolation of *Listeria* species

3.5.3.1. Enrichment and culture

The ISO 11920-1 (1996) method for qualitative isolation and identification of *Listeria* species was used as described by Indrawattana *et al.*, (2011) with modifications. Ten (10) gram or 10 ml of each sample was aseptically weighed and transferred into a properly labeled stomacher bag containing 90 ml of *Listeria* Enrichment Broth (Oxoid formulation) and homogenised with the Stomacher 400 Lab blender for 1 minute. The homogenate was incubated at 37°C for 24 hours aerobically (Gulmez and Guven, 2003). Then 0.1 ml of each enriched culture was streaked on to *Listeria* selective agar (Oxford formulation) (CM0856)

and incubated at 37°C for 24-48 hours. Plates were examined for typical *Listeria* colonies viz grayish colonies surrounded by black halos with sunken center (Hitchins, 1995; Aygun and Pehlivanlar, 2006). Colonies showing black to brown colonies surrounded by black halos with sunken center on *Listeria* selective agar were sub-cultured onto Tryptone Soya Agar (Oxoid, M290) supplemented by 0.6% of Yeast Extract Powder (Oxoid, LP0021) (TSYEA) and incubated at 37°C for 24 hours. The following tests were used for confirmation: Gram staining, motility and catalase test, sugar fermentation, oxidase, aesculin hydrolysis.

3.5.4 Biochemical characterization of isolates

A grayish colony surrounded by a black halo was further tested for β -haemolysis on 7% sheep blood agar and carbohydrate fermentation using mannitol, rhamnose and xylose as described by Janzten *et al.*, (2006), and Jemmi and Stephan (2006). Other tests carried out included motility test, aesculin hydrolysis and oxidase test.

3.5.4.1 Catalase test

A drop of 3% hydrogen peroxide (H_2O_2) was placed on a glass slide, a bit of growth was removed from *Listeria* Selective Agar with a wire loop. The evolution of gas (oxygen) was indicated by formation of bubbles on the glass slides and also indicated positive result

3.5.4.2 Gram's stain

Gram staining was done using suspected *Listeria* species colonies on *Listeria* selective agar plates. A pure colony of *Listeria* was emulsified in normal saline on the slide to form a smear. The smear was allowed to air dry completely. The slide, (with the smear uppermost) was fixed by rapidly passing through it Bunsen burner flame in two quick succession. The

smear was allowed to cool. The fixed smear was covered with crystal violet stain for 30 seconds-1minute. The stain was rapidly washed off with clean slow running tap water. The smear was again covered with Lugol's iodine for another 30 seconds-1minute and washed off with clean tap water and was rapidly decolourized (few seconds) with acetone-alcohol. This was washed immediately with clean tap water. The smear was then covered with Safranin dye for 2 minutes and washed off with clean tap water. The stained slide was placed in a draining rack and the smear allowed to air dry. The smear was examined microscopically using oil immersion objective lens ($\times 100$).

Listeria species that appeared purple to blue in the Gram-stained slides were tentatively confirmed due to their Gram-positive stain morphology viz rods, arranged singly, in short chains, in pairs at V-form angles and in groups that were parallel to each other along the long axis. The results of the Gram reactions were recorded accordingly.

3.5.4.3 Motility test

The motility medium (1% Nutrient agar) was inoculated with the suspected colonies by making a fine stab at the centre of the motility medium with an inoculating pin to a depth 1-2cm of the bottom of the tube and incubated at 25⁰C (room temperature) for 24 hours to 48 hours. At about 0.5 cm below the surface of the agar, a layer of increased growth was observed, like an umbrella signifying that the presumptive isolates were motile. In this zone of reduced oxygen tension presumptive isolates showed a better development than under aerobic or strictly anaerobic conditions (Quinn *et al.*, 1994).

3.5.4.4 Sugar fermentation testing

The *Listeria* isolates to be tested were inoculated into a sterile peptone water and incubated at 37°C for 2-3 hours. One drop (0.5ml) of each isolate from peptone water was used to inoculate the sugars. After inoculation, all the bijou bottles inoculated with *Listeria* organisms were incubated at 37°C and examined at an interval of 24hrs and 48hrs. Fermentation test reactions indicated by colour change were recorded. A colour change from light pink to yellow indicated that fermentation had occurred thus a positive result (Collee and Miles, 1989).

3.5.4.5 Haemolysis

Seven percent sheepblood agar base (Oxoid, CM0854) was used to determine the presence of listeriolysin (LLO) exotoxin in *L.monocytogenes* and *L. ivanovii* strains. The presumptive *Listeria* isolates were inoculated onto 7% sheep blood agar and incubated at 37°C for 24hours. After 24hours, the 7% sheep blood agar plates were observed for α and β haemolysis. β haemolysis presented with a clear around the while α haemolysis presented with a greenish colouration around the presumptive colonies (Morobe *et.al.*, 2009).

3.5.4.6 Aesculin hydrolysis

The isolates were grown on bile aesculin agar for 24hours. A positive result was indicated by black colouration of the whole medium, after 24hours of incubation, a negative reaction indicated by a lack of colour change throughout the medium.

3.5.4.7 Oxidase test

Pieces of filter papers were wetted with a few drops of a dilute solution (about 1%) of the oxidase reagent, tetra methyl-p-phenylene diamine dihydrochloride. A loopful of the presumptive colony from Bile Aesculin Agar with a wire loop and was smeared on the wet

paper. The development of an intense purple colouration by bacterial cells in the smear within 10 – 15seconds were recorded as positive test.

3.6 Confirmatory Test using Microgen™*Listeria*- ID system

Prior to inoculation into the Microgen *Listeria* ID, isolates were checked to ensure they are *Listeria* species (Short Gram positive bacillus, motile at 25⁰C, catalase positive and oxidase negative) by subjecting them to conventional biochemical tests namely catalase test, aesculin hydrolysis test, haemolysis, oxidase test and sugar fermentation test. The suspending broth was brought to room temperature after storage at -4⁰C before inoculation of micro well test strips. A single well – isolated colony from an 18-24hours culture was selected and emulsified in a vial of *Listeria* suspending medium (2.5ml). The medium was mixed thoroughly until a homogenous medium was obtained. Micro well test strips were removed from the foil pouch, placed on the holding frame and the lid removed. Using a sterile Pasteur pipette, 4 drops (approximately 100µL) of the bacterial suspension was transferred to each well of the microwell test strips.

As a purity check, 1 drop of the organism suspension was transferred onto nutrient agar plate. The plate was incubated aerobically at 35-37⁰C for 18-24 hours. One drop of the haemolysin reagent was added to well 12 and the lid was replaced onto the microwell test strip and incubated at 37^o C for 24hours. After incubation, lids were removed from the microwell test strip and the result was recorded on the report form provided. The various octal codes were ran on Microgen ID software version 1.2.5.26 to obtain various species of *Listeria*

3.7 Antibiotic Susceptibility Testing

The antibiotic susceptibility of the isolates was determined by Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (Oxoid, Basingstoke, UK). The antibiotics that were tested included: Sulfamethoxazole-Trimethoprim (25 µg), Amoxicillin (10µg), Tetracycline (30µg), Erythromycin (15 µg), Gentamicin (30 µg), Chloramphenicol (30 µg) Ciprofloxacin (5 µg), Streptomycin (10 µg), Cefoxitin(30 µg), Nitrofurantoin (50µg), Trimethoprim (30µg) and Vancomycin (30 µg). Three to five well isolated colonies of *Listeria were* transferred into 5 mL Brain Heart Infusion broth (BHIB, Oxoid) and incubated at 37°C for 18 to 24 h. The overnight broth culture was diluted using sterile distilled water to a turbidity equivalent to 0.5 McFarland standard (Harakeh *et al.*, 2005) (approximately 108 cfu/ml), and inoculated onto the entire surface of a dried Mueller-Hinton Agar (MHA, Oxoid) plate creating a lawn of the culture. The inoculated MHA plates was allowed to dry at room temperature before placing the antibiotic discs followed by incubation. After incubation for 24 h at 37°C, the diameter (in mm) of the zone around each disk was measured and interpreted in accordance with the Clinical and Laboratory Standards Institute Standards guidelines (CLSI, 2006) using *Staphylococcus aureus* as control strain. The multiple antibiotic resistance (MAR) index was determined for each of the isolates. MAR index was calculated by dividing the number of antibiotics each isolate was resistant to (a) by the total number of antibiotics to which the isolates were tested (b) (Singh *et al.*, 2010)

$$\text{MAR index} = a/b$$

3.8

Listeria monocytogenes Identification by PCR

3.8.1. DNA extraction

3.8.1.1 Phenol/chloroform DNA extraction method

The *Listeria* suspensions were dissolved by addition of 400µL lysing buffer (Sigma, USA), 25µL 20Mgml⁻¹proteinase K and were vortexed for 20seconds. After incubation at 65°C for 1hour in a heating block, 50µL phenol: chloroform: isoamylalcohol (Merck, Germany) was added and vortexed for 15seconds after which it was centrifuged at 13,000rpm for 10minute. Aqueous phase was transferred to another new Eppendorf tube and added 400µl chloroform and vortexed for 10seconds. The aqueous phase was further transferred to new Eppendorf tube, 100µL absolute ethanol and 40µL 3M sodium acetate were added and were inverted gently 3time until DNA precipitated and stored at – 20°C overnight. After storage, DNA was dried and washed by dipping the end of the pipette into 1ml 70ml (Merck, Germany) for 30s before dissolving in ultra clean pure water (Simon *et al.*, 1996).

3.8.2 The primers

Haemolysin (*hyl* A) gene was detected using the Primers (LM1: 5'- CCTAAGACGCCAATCGAA -3' (Forward) and LM2: 5'- AAGCGCTTGCAACTGCTC - 3' (Reverse) (Lakicevic *et al.*, 2010) was used to target *hylA* gene which codes for Listeriolysin O (A protein which helps them to escape the phagosome) and an expected amplicon of 702 bp was obtained

3.8.3 PCR amplification procedure

PCR was performed in a final volume of 50µl micro-tubes containing 1xPCR buffer (10xPCR buffe: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM MgCl₂, 200 µM of each dNTP, 2.5 µM of each primer, 1 U of Taq polymerase (promega) and 1 µg of DNA template. The samples was amplified in a DNA thermal cycler (MJ Research Inc.)

with forward and reverse primers complementary to the *hyl A* gene. Reaction mixture with distilled water and known *L. monocytogenes* isolate were incorporated as negative and positive control, respectively in each run. Amplification was carried out using 35 cycles of amplification at 94°C for 5 minutes (initial denaturation), 94°C for 30 seconds (final denaturing); 55°C for 30 seconds (annealing), and 72°C for 1 minute (initial extension), this reaction was followed by 5 minutes of an additional extension at 72°C.

3.8.4 Electrophoresis of PCR product

PCR products were separated on 1.5% agarose gel at 100V for 40 minutes in Tris-acetate buffer, visualized by staining with 0.5 µg/ml ethidium bromide and illuminated by UV-trans illuminator and documented by a gel documentation apparatus. A 1500 bp DNA ladder with a ladder increment of 100bp (Fermentas, Waltham, Massachusetts, USA) was used as a size reference for PCR assay

3.9 Data Analyses

Data was subjected to descriptive statistics using Statistical Package for Social Science (SPSS) version 20.0 (Irener *et al.*, 2013). Prevalence was calculated using the formula below. Prevalence was compared with the occurrence of the organism with type of birds, sample location using Chi-square.

$$\text{Formula: Prevalence} = \frac{\text{Number of positive samples}}{\text{Total number of samples collected}} \times 100$$

CHAPTER FOUR

RESULTS

4.1 Contamination of Chicken Carcasses and Poultry Processing Environment with *Listeria* Species in Zaria, Nigeria.

Based on the growth on *Listeria* selective agar, Gram stain reaction, oxidase test, motility test, aesculin hydrolysis, haemolysis, sugar fermentation tests and catalase test, a total of 168 (40.77%) suspected *Listeria* spp. were isolated from 412 samples of raw fresh chicken carcasses. The isolation was distributed between five species in Zaria, Nigeria: *Listeria ivanovii* 58 (59.18%), *Listeria monocytogenes* 10 (10.20%), *Listeria grayi* 17 (17.34%), *Listeria welshimeri* 12 (12.24%) and *Listeria innocua* 1(1.02) (Table 4.1).

Table 4.1: Differentiation of *Listeria* species based on conventional biochemical tests

| Species | Freq | Haem | Cata | Moti | Xylo | Rham | Mann | Oxid | Aescu |
|----------------------------|------|------|------|------|------|------|------|------|-------|
| <i>L. ivanovii</i> 58 | + | + | + | - | - | + | + | | |
| <i>L. grayi</i> 17 | ++ | - | +/- | + | + | | | | |
| <i>L. monocytogenes</i> 10 | + | + | - | + | - | + | + | | |
| <i>L. welshimeri</i> 12 | + | ++ | +/- | - | + | | | | |
| <i>L. innocua</i> 1 | - | + | + | - | +/- | + | + | | |

Key: Haem: haemolysis, Cata: catalase test, Moti: motility, Xylo: xylose, Rham: rhamnose, Mann: mannitol, Oxid: oxidase test, Aescu hydro: aesculin hydrolysis, +: Positive, +/-: variable, -: negative, Freq: frequency.

Of the 98 isolates that were subjected to Microgen™ *Listeria* - ID System (Microgen bioproducts) to confirm isolates from conventional biochemical tests. 39 out of the 40 isolates met the 90% cut-off point criteria for species identification by the Microgen™ *Listeria*-ID System. One of the isolates (*L. grayi*) gave 42.16% which is below the 90% cut off point for probable identification while the remaining 39 isolates had above 95% level of probability. Distribution of the 40 (100%) *Listeria* species based on Microgen™ *Listeria* -ID system showed that 4 (10%) were *L. grayi*, 2 (5%) were *L. welshimeri*, 2 (5%) were *Listeria monocytogenes* and 32 (80%) were *L. ivanovii* (Table 4.2)

Table 4.2: Frequency of *Listeria* species from chicken carcasses and poultry processing environment in Zaria, Nigeria Based on Microgen™ *Listeria* –ID system.

N=412

| Species | Frequency (%) |
|-------------------------|----------------------|
| <i>L. grayi</i> | 4(10) |
| <i>L. ivanovii</i> | 32(80) |
| <i>L. monocytogenes</i> | 2(5) |
| <i>L. welshimeri</i> | 2(5) |
| Total | 40 (100) |

Key: N: Total number of numberof samples collected

Among the chicken carcasses, broiler carcasses had the highest prevalence of 13(8.44%) when compared with local chicken carcasses which had a prevalence value of 12 (7.79%). Distribution of *Listeria* species in chicken carcasses showed that broiler carcasses had more *L. ivanovii* isolates compared to local chicken carcasses with 8.44% and 6.41% respectively. *L. grayi* was isolated from floor 1 (3.84%), bowl 2(7.69%) and from local chicken carcasses 1(3.84%). Similarly, Local chicken carcasses was contaminated with only 1 (0.64%) *L. welshimeri* isolate. *L. monocytogenes* was detected from 2 (1.30%) broiler carcasses (Table 4.3)

Distribution of *Listeria* species by sample types from poultry processing environment showed that bowl samples had the highest prevalence of *Listeria* species with 7 (26.92%) of the samples positive followed by floor 6(23.08%). Table and knife swab samples have the least *Listeria* contamination with 1 (3.85%) of each contaminated with *Listeria* species (Table 4.4).

Listeria ivanovii was the most commonly found species with 32 (7.77%) of the samples positive for *L. ivanovii* with most of the isolates recovered from the floor 5 (15.38%) while 4 (15.38%) were from bowl and 1 (3.84%) each were from knives and tables

Table4.3: Prevalence and distribution of *Listeria* species in chicken carcasses in Zaria, Nigeria.

| Sample type | No of samples tested | Total(%) number positive for <i>Listeria</i> spp. | | | | |
|-------------------------|----------------------|---|-----------------|----------------------|-------------------------|---------|
| | | <i>L. ivanovii</i> | <i>L. grayi</i> | <i>L. welshimeri</i> | <i>L. monocytogenes</i> | |
| Local chicken carcasses | 154 | 12(7.79) | 10(6.49) | 1(0.64) | 1(0.64) | 0 (0) |
| Broiler carcasses | 154 | 13(8.44) | 11(7.14) | 0(0) | 0(0) | 2(1.29) |
| Total | 308 | 25(8.11) | 21(6.81) | 1(0.32) | 1(0.32) | 2(0.64) |

Table 4.4: Prevalence and distribution of *Listeria* species in poultry processing environment in Zaria, Nigeria.

| Sample type | No of samples tested | | | | | |
|-------------|----------------------|---|--------------------|-----------------|----------------------|-------------------------|
| | | Total(%) number positive for <i>Listeria</i> spp. | <i>L. ivanovii</i> | <i>L. grayi</i> | <i>L. welshimeri</i> | <i>L. monocytogenes</i> |
| Table swab | 26 | 1(3.84) | 1(3.84) | 0(0) | 0(0) | 0(0) |
| Floor swab | 26 | 6(23.07) | 5(19.23) | 1(3.84) | 0(0) | 0(0) |
| Knife swab | 26 | 1(3.84) | 1(3.84) | 0(0) | 0(0) | 0(0) |
| Bowl | 26 | 7(26.92) | 4(15.38) | 2(7.69) | 1(3.84) | 0(0) |
| Total | 104 | 15(14.42) | 11(10.57) | 3(2.88) | 1(0.96) | 0(0) |

Distribution of *Listeria* isolates by location showed that Sabo market had the highest Prevalence of *Listeria* species with 15(14.56 %) of the samples positive followed closely by Samaru 11 (10.68%) and Danmagaji with 11 (10.68) and 10 (9.71%) respectively. The least contamination with *Listeria* species was observed at Tudunwada live birds' market with a prevalence of 4 (3.88%) (Table 4.5).

Among the *Listeria* species isolates recovered from the four markets, *Listeria ivanovii* was the most commonly contaminating species, with 32 (7.77%) of the samples positive for *L. ivanovii* among other *Listeria* species. Sabo live birds' market had the highest prevalence of *Listeria ivanovii* isolates with 15 (14.56%) and the least prevalence of *Listeria ivanovii* with 2 (1.945%) was recorded at Tudunwada live birds' market. 2 *Listeria grayii* isolates were recovered from Tudunwada while 1 (0.97%) each was recovered from Danmagaji and Samaru respectively. Similarly, only 2 *Listeria welshimerii* isolates were also recovered from Danmagaji live birds' markets (Table 4.5)

Table 4.5: Prevalence and distribution of *Listeria* species by location of chicken carcasses and poultry processing environment in Zaria, Nigeria

| Location | No of samples tested | Number and percentages (%) of positive samples | | | | |
|-----------|----------------------|--|--------------------|-----------------|----------------------|-------------------------|
| | | <i>Listeria</i> species | <i>L. ivanovii</i> | <i>L. grayi</i> | <i>L. welshimeri</i> | <i>L. monocytogenes</i> |
| Danmagaji | 103 | 10(9.71) | 7 (6.79) | 1 (0.97) | 1 (0.97) | 1(0.97) |
| Sabo | 103 | 15(14.56) | 13 (12.62) | 0 (0) | 1 (0.97) | 1(0.97) |
| Samaru | 103 | 11(10.68) | 10 (9.71) | 1(0.97) | 0 (0) | 0(0) |
| T/wada | 103 | 4 (3.88) | 2 (1.94) | 2 (1.94) | 0 (0) | 0(0) |
| Total | 412 | 40(9.71) | 32(7.77) | 4(0.97) | 2(0.49) | 2(0.49) |

4.2

Antibiotic Resistance of *Listeria* Species

From the percentage distribution of antibiotics susceptibility of the 40 *Listeria* isolates to 12 antimicrobial agents, all the 40 *Listeria* isolates were resistant (100%) to amoxicillin, erythromycin, tetracycline and vancomycin. Similarly, the isolates also showed resistance to the following antimicrobial agents to varying degrees, Trimethoprim (97.5%), sulfamethoxazole-trimethoprim (95%), streptomycin (92%) and chloramphenicol (72%). The isolates showed least resistance to nitrofurantoin, gentamicin and ciprofloxacin at the percentages 42.5%, 15% and 30% respectively (Figure 4.2).

TE, S, F, AML, VA, C, E, W, STX was the most common pattern occurring in 19 of the 40 isolates, alone (8 isolates) or with one (5 isolates) or two (6 isolates). Thirty three of the 40 multidrug resistant isolates has resistance profile combinations containing TE, aminoglycosides and sulphonamides (Table 4.6).

The mean MAR index was 0.75 ± 0.066 with minimum being 0.50 and maximum 0.92. All the 40 isolates had multi-antibiotic resistant index above the bench of 0.20 (Table 4.7).

Thirty eight *Listeria* isolates showed resistance to 8 or more antimicrobial agents while 1 isolate each showed resistant to 5 and 7 antimicrobial agents respectively (Table 4.8).

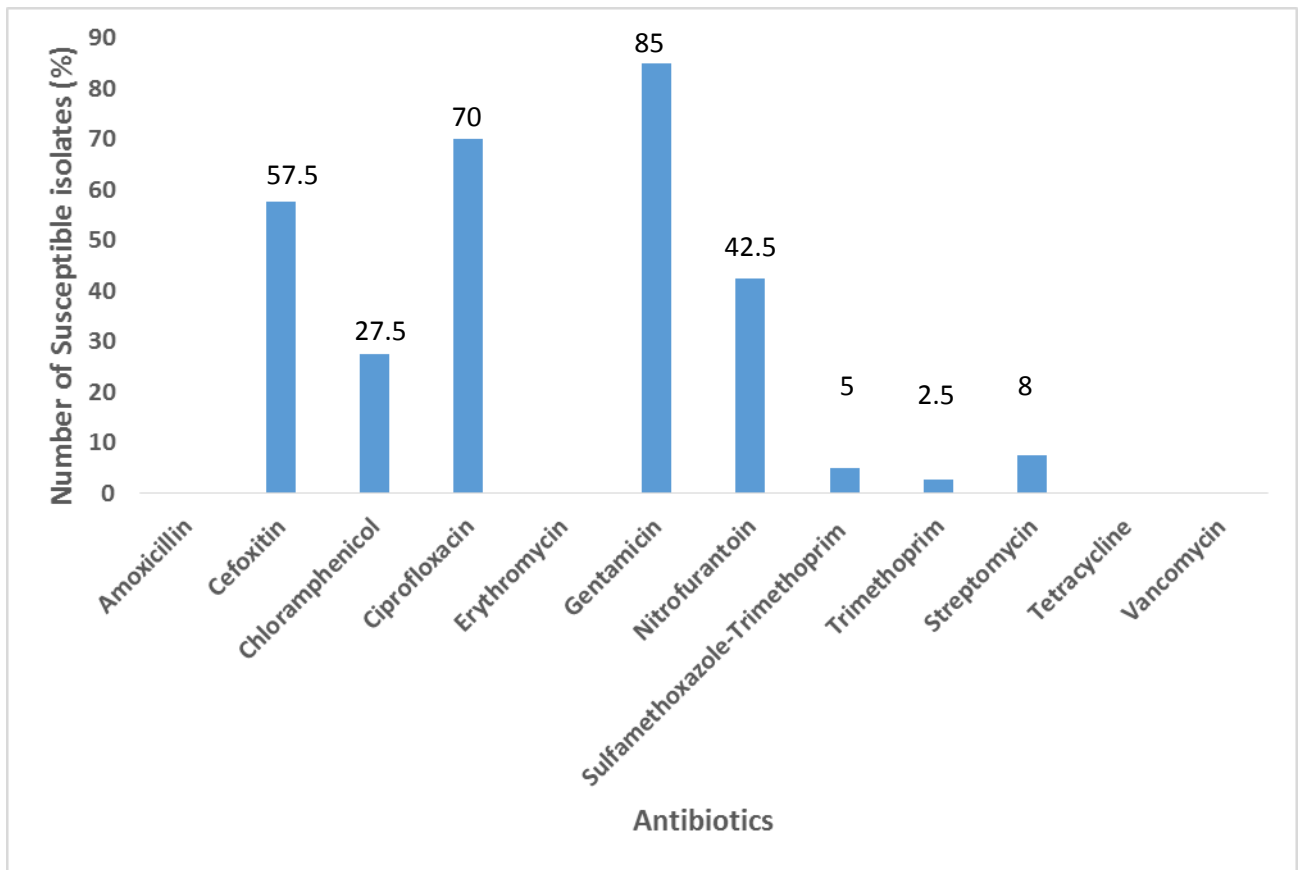


Figure 4.1: Percentage distribution of antibiotics susceptibility test of the 40 isolates of *Listeria* species to 12 different antibiotics

Table 4.6 Antimicrobial resistance patterns of 40 *Listeria* species isolates in chicken carcasses and poultry processing environment in Zaria, Nigeria

| S/N | Pattern | Frequency |
|-----|---------------------------------------|-----------|
| 1. | TE, S,F, AML, VA, C, E, W, STX | 8 |
| 2. | TE, S, F, AML, FOX, VA, C, E, W, STX | 6 |
| 3. | TE, S,F, CIP, AML,VA,C,E,W,STX | 5 |
| 4. | TE, S, F, AML, VA, E, W, STX | 5 |
| 5. | TE, S, CIP, AML,VA,C,E,W,STX | 2 |
| 6. | TE, S,F,AML,FOX,VA,E,W,STX | 2 |
| 7. | TE, F, AML,VA,C,E,W,STX | 1 |
| 8. | TE,S,F,CIP,AML,FOX,VA,C,E,W | 1 |
| 9. | TE,S,F,CIP,AML,FOX,VA,E,W,STX | 1 |
| 10. | TE,S,AML,FOX,VA,C,E,W,STX | 1 |
| 11. | TE,CN,S,AML, VA, C,E,W,STX | 1 |
| 12. | TE, S, AML,VA,E | 1 |
| 13. | TE,CN,S, CIP, AML,VA, C, E,W,STX | 1 |
| 14. | TE, CN, S, AML, FOX, VA, C, E, W, STX | 1 |
| 15. | TE, S,AML,FOX,VA,C,E,W | 1 |
| 16. | TE,S,AML,VA,E,W,STX | 1 |
| 17. | TE, CIP,AML,FOX,VA,C,E,W,STX | 1 |
| 18. | TE, CN, S, CIP,AML,FOX,VA,C,E,W, STX | 1 |

Key: AML, Amoxicillin; FOX, Cefoxitin; C, Chloramphenicol; CIP, Ciprofloxacin; E, Erythromycin; CN, Gentamicin; F, Nitrofurantoin; STX, Sulfamethoxazole-Trimethoprim; W, Trimethoprim; S, Streptomycin; TE, Tetracycline; VA, Vancomycin

Table 4.7: Multi-drug resistant index of the 40 *Listeria* species isolates obtained from chicken carcasses and poultry processing environment in Zaria, Nigeria

| S/N | Isolates (a/b) | Frequency | Number of Antibiotics to which the isolate was resistant to (a) | MAR Index |
|-----|--|-----------|--|--------------|
| 1. | SMLC 139, DF 57, SBBC87 | 3 | 11 | 0.92 |
| 2. | DB84, SBLC 85, SBBC 91, DLC59 DB85, SBF67, SBBC78, SMB94 TB78, SBLC112, TF59, SBBC84 | 12 | 10 | 0.83 |
| 3. | SMBC 131, SMBC 128, SBBC80, DLC49 DB83, DLC47, SBBC83, SBBC84, SBF61 DB82, TK6, SMF 74, DF58 | 13 | 9 | 0.75 |
| 4. | DT 33 , SMBC 120, SMLC 155, SBLC 116 SBLC 83, SMLC 149, SMBC 132, SMBC 120 SBBC88, | 9 | 8 | 0.67 |
| 5. | SBBC 108, TB 77 | 2 | 7 | 0.58 |
| 6. | SMLC 122, | 1 | 6 | 0.50 |

b = Total number of antibiotics used, 12

Key

| | |
|-------|---|
| SMLC: | Local chicken carcass from Samaru market |
| DF: | Floor swab from poultry processing environment in Danmagaji |
| SBBC: | Broiler carcass from Sabo market |
| DLC: | Local chicken carcass from Danmagaji |
| DT: | Table swab from poultry processing environment |
| SMBC: | Broiler carcass from Samaru market |
| DB: | Bowl swab from poultry processing environment in Danmagaji |
| SBF: | Floor swab from poultry processing environment in Sabo |
| SMB: | Bowl swab from poultry processing environment in Samaru |
| TF: | Table swab from poultry processing environment in Tudunwada |
| TK: | Knife swab from poultry processing environment in Tudunwada |
| SMF: | Floor swab from poultry processing environment in Samaru |
| TB: | Bowl swab from poultry processing environment in Tudunwada |

4.3

PCR Assay for the Identification *Listeria* Species

All the *Listeria* species isolates were screened for the presence of *hyl A* genes. Only *Listeria monocytogenes* isolates gave a band at 702 bp which is an indicator for the presence of *hyl A* and further confirmed the *L. monocytogenes*. Gel electrophoresis of the amplified *hyl A* gene (Fig 4.3 and 4.4), Lane 1-6 and Lane 1-8 represent *Listeria* isolates, M represents the molecular weight (100bp) marker while C- and C+ are the negative and positive controls (*Listeria monocytogenes*). Only two of the isolates were to harbour *hyl A* gene (SBBC 116 and DBC 47). In figure 4.3, well 1 contained *Listeria monocytogenes*(SBBC 116) isolate which gave a DNA fragment size of 702bp when compared with the molecular marker (100bp) on gel electrophoresis visualized under the UV light similarly, figure 4.4, well 2 (DBC 47) which contained the second *L. monocytogenes* isolate also gave a band of an amplicon size of 702bp (is the size of *hyl A* gene). Distribution of these genes by location showed 1 of the two isolates (DBC 47) came from Danmagaji and the other came from Sabo (SBBC 116) while distribution of these genes by sample type showed that these genes were found in broiler carcasses and only in *L. monocytogenes*.

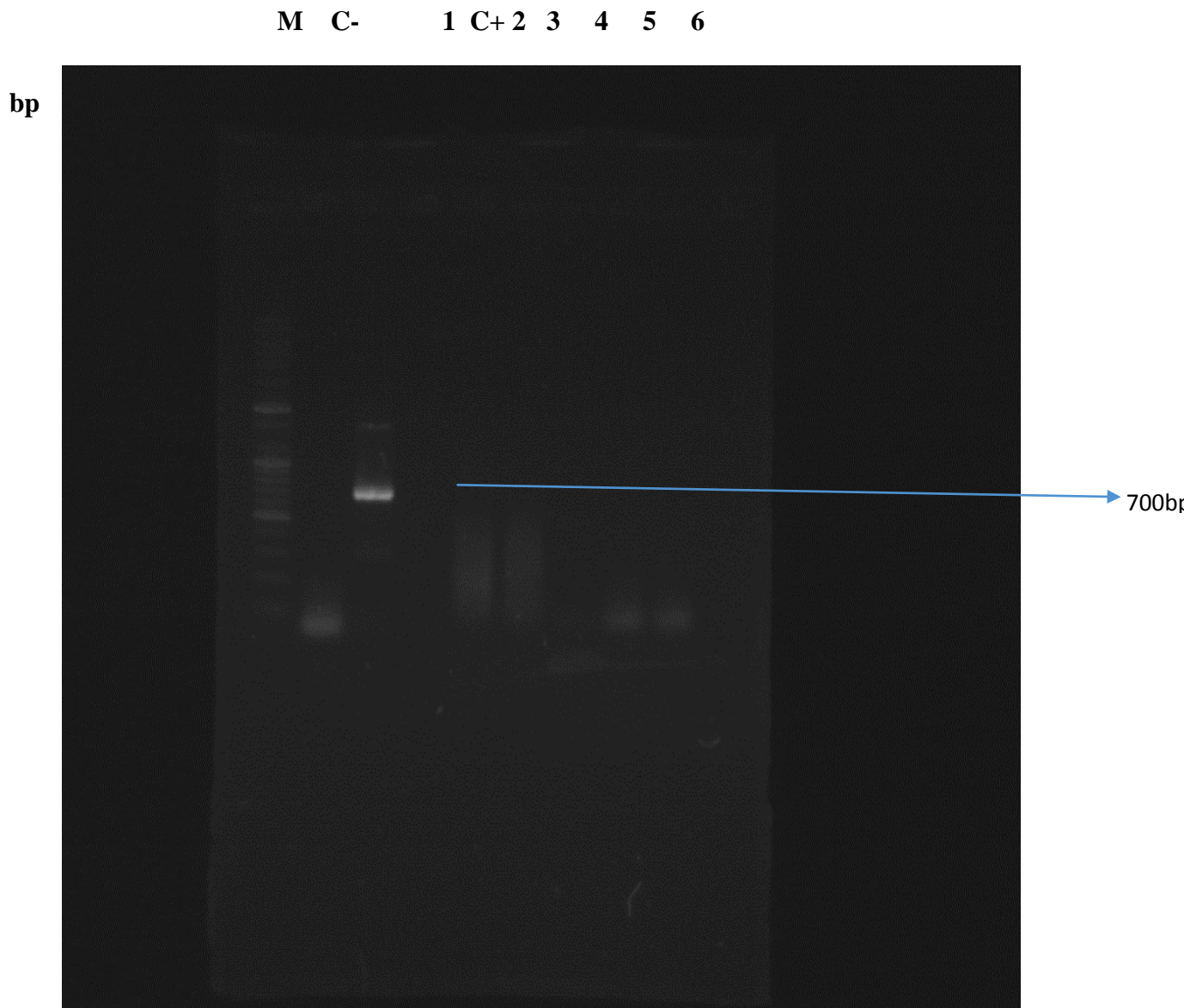


Plate I: Agarose gel electrophoresis of *hlyA* gene of *Listeria monocytogenes* isolate (lane 1-6); 2-6- other species of *Listeria*, C-: Negative control, C+: Positive control. One hundred –bp ladder (Lane M) was used as a molecular marker

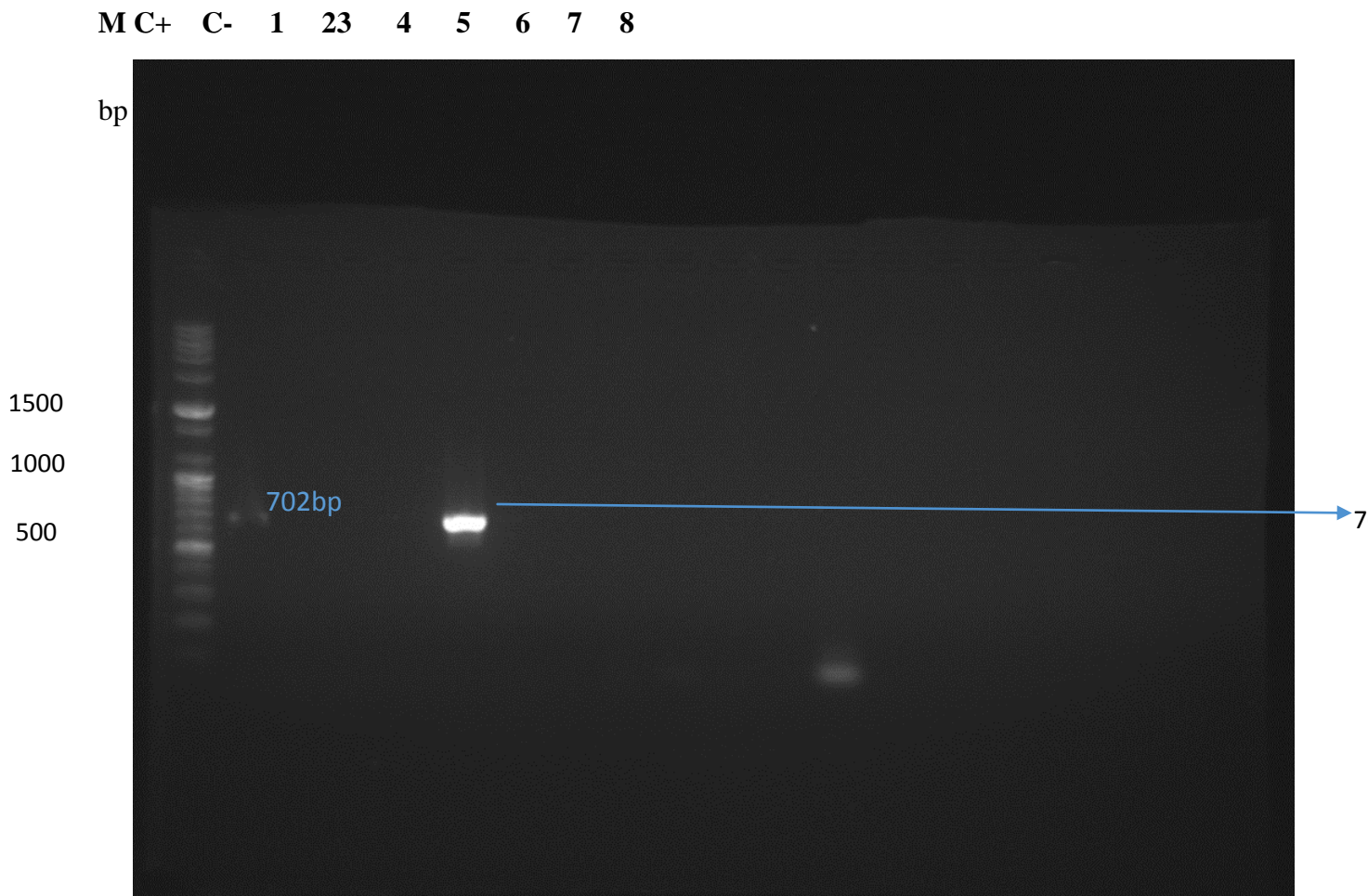


Plate II: Agarose gel electrophoresis of *hylA* gene amplicon specific for *L. monocytogenes* by polymerase chain reaction method. Lanes M (100 bp ladder); C+: (positive control), C-: (Negative control), Lane 1 to 8 are representative run for PCR results of 8 extracted DNA. Lane 2 showing positive and negative (lanes 1, 3, 4, 5, 6, 7, 8) amplicon *hylA* gene.

CHAPTER FIVE

DISCUSSION

Chicken have been frequently contaminated with *Listeria* species and may serve as vehicles of other pathogenic organisms. The frequent occurrence of *Listeria* species especially *Listeria monocytogenes* in meat and chicken may pose a potential risk for consumers (Mahmood *et al.*, 2003).

This study established an overall prevalence of 9.71% of *Listeria* species in chicken carcasses and poultry processing environment in Zaria, Nigeria. This study also established prevalence values of 8.12% in raw chicken carcasses and 14.42% in poultry processing environment respectively. Similar prevalence of 6.67% of *Listeria* in raw chicken carcasses in 3 Markets was reported in Markurdi, Nigeria (Daniel *et al.*, (2015). These findings are also similar to prevalence reported in Egypt by Alsheikh *et al.*, in 2012 of 13.6% and Hudson *et al.*, (1992) of 12.5% in New Zealand and 13.7% by Uyttendaele *et al.*, (1999) in Belgium. Similarly, Ashraf *et al.* (2010) recovered *Listeria* spp. from chicken meat samples in Egypt and Ikeh *et al.*, (2010) in Nigeria isolated *L. monocytogenes*, *L. murrayi grayi* and *L. seeligeri*, *L. ivanovii*, *L. innocua*, and *L. welshimeri* (Daniel *et al.*, 2015). Other researchers (Jalali and Abedi, 2007; Peckrovi *et al.*, 2008) reported that *L. monocytogenes* was not detected from raw chicken carcasses samples by either conventional method or PCR technique using primers targeting the (*hlyA*) gene in Iran and Croatia, respectively. Other studies (Mena *et al.*, 2004; 2010; Yucel *et al.*, 2005) confirmed that *L. monocytogenes* was recovered in high percentages (36.1, 60, 11.5 and 21.6%) from raw chicken in Spain, Portugal, Turkey and Italy, respectively using conventional and serotyping methods. The difference in prevalence when compared to other studies could be attributed to usage of different sample sizes, isolation techniques, compliance to good hygienic practices and geographical locations.

The overall prevalence of *Listeria* species in this study is an indication of poor hygiene level in the processing and handling of chicken carcasses by butchers and also, it could be as a result of poor sanitary conditions in chicken processing environment. The implication of this is that chicken carcasses offered to consumers in Zaria are contaminated and of public health concern.

Prevalence of *Listeria* spp. by sample types showed that broiler carcasses had a prevalence of 8.33% which is similar to 9.2% prevalence reported by Ckukwu *et al.*, (2006) from smoked fish in Jos, Nigeria and the local chicken carcasses had a prevalence of 7.69% which is lower than the prevalence of 34.4% of *Listeria* spp. in raw chicken and RTE meat products, in Trinidad reported by Gibbons *et al.*, (2006). This could be due to a better hygienic condition in Zaria, Nigeria.

The prevalence of *Listeria* species in poultry processing environment in Zaria was 14.42%. This prevalence could be attributed to contamination of poultry processing environment which could either be as a result of humans or environmental factors. Chicken handlers could have contributed to the contamination of poultry processing by their practices: use of contaminated water to rinse chicken carcasses after defeathering, not maintain good hand hygiene, use of dirty utensil in the processing and handling of chicken carcasses, mixing intestinal contents with dressed carcasses and unrestricted access into poultry processing environment. Contamination of poultry processing could also emanate from the environment, soil particles in the poultry processing environment could also be responsible for chicken carcasses contamination because soil is a reservoir of *Listeria* species.

Biochemical tests both conventional and MicrogenTM *Listeria*- ID system were used to confirm the presumptive *Listeria* colonies from *Listeria* selective agar. These tests played a

key role in the confirmation of the 40 *Listeria* isolates which were used to establish the various prevalence values in this study.

The overall prevalence of 9.71% of *Listeria* species in chicken carcasses and poultry processing environment is of significance in the epidemiology of *Listeria* disease because of the possibility of these organisms spreading to other geographical locations which are hitherto free these organisms.

This study has demonstrated that the highest occurrence among *Listeria* spp. was *L. ivanovii* in the tested samples (7.76%) followed by *L. grayi* (0.97%), *L. monocytogenes* (0.48%) and *L. welshimeri* (0.48%). This agrees with Osaili *et al.*, (2011) who reported that *L. ivanovii* was the predominant isolate among *Listeria* spp. in fresh dressed broiler chicken samples (30%). Chicken carcasses can become contaminated with *Listeria* spp. either environmentally or from healthy carrier birds during production in the farm (Skovgaard and Morgen, 1988). In poultry abattoir and processing plant, improper cleaning and disinfecting of environment and equipment and also mishandling of the products may lead to *Listeria* contamination of poultry carcasses and the final products (Uyttendaele, *et al.*, 1997). Contamination of ready-to-eat poultry products can be occurred after cooking by cross-contamination either environmentally or via workers, surfaces and equipment (Osaili, *et al.*, 2011). In addition, the ability of *L. monocytogenes* to form biofilms inside food facility and on the surfaces of food production lines that is quite difficult to be removed and cleaned (Bresford *et al.*, 2001). For example, *Listeria* spp., especially *L. monocytogenes* is able to transfer through vacuum and gas-packaged products due to their ability.

Listeriosis has commonly been treated with penicillin or ampicillin in combination with aminoglycoside (Charpentier and Courvalin, 1999), while alternative treatments included

tetracycline, erythromycin or chloramphenicol, alone or in combination (Hof, 1991). Currently a combination of ampicillin and gentamicin is standard therapy for systemic listeriosis, and trimethoprim-sulfamethoxazole (TMP-SMX) may be used for patients with beta-lactam intolerance (Schlech, 2000). Although *Listeria* spp. have been reported to be susceptible to antibiotics (Hof, 1991), more recent reports have indicated resistance in *Listeria* spp. to antibiotics active against Gram-positive bacteria (Abraham *et al.*, 1998). This development of antibiotic resistance among *Listeria* spp. shows a similar pattern world-wide and is on the increase (Walsh *et al.*, 2001). Antibiotic resistance in *Listeria* species is due to the acquisition of three type mobile genetic elements: self-transferable and mobilizable plasmids and conjugative transposons (Charpentier *et al.*, 1995).

In this study, resistance of the isolates against erythromycin (64.3%) is contrary to that obtained by Morobe *et al.*, (2009) in Botswana where all the isolates from various food isolates were reported to be susceptible to erythromycin but similar to the findings of Ennaji *et al.*, (2008) and Aurelli *et al.*, (2000). Resistance to sulfamethoxazole-trimethoprim (95%) and vancomycin (100%) were found to be high as compared to 17.1% for sulfamethoxazole-trimethoprim and 2.4% for Vancomycin obtained by (Wong *et al.*, 2012) as well as 29.82% for sulfamethoxazole-trimethoprim obtained by (Morobe *et al.*, 2009). There was also resistance to amoxicillin by *Listeria* isolates in this study, Daniel *et al.*, (2015) also reported resistance to amoxicillin by *Listeria* from chicken carcasses. Low resistance of *L. monocytogenes* to ciprofloxacin and gentamicin seen in this study is similar to reports by Yakubu *et al.*, (2012a) and Rahimi *et al.*, (2012) where over 80.0% of isolates from various sources were found to be susceptible to each of these antimicrobial agents.

Gentamicin is the drug of choice in the treatment of listeriosis, this finding offers glimpse of hope incase of an outbreak of listeriosis. For chloramphenicol, all the isolates were resistant (100%) to this antimicrobial agent which contrary to the findings of Yucel *et al.*,(2005) who reported that *L. monocytogenes* isolated from meat products were highly sensitive with a percentage of (100%) to chloramphenicol. This difference in susceptibility pattern of *Listeria* to chloramphenicol could be attributed to heavy usage or abuse of this antimicrobial agent in poultry either as feed supplement of in the treatment of sick birds

High levels of antimicrobial resistance that were encountered in the study may be attributed to their frequent abuse in poultry and in the treatment of various human diseases in Nigeria. Furthermore, resistance to tetracycline may develop as a result of using this drug as a food supplement in animal feed and as a drug of second choice in the treatment of human diseases and resistance to this drug has been previously reported in environmental isolates (Prazak *et al.*, 2002; Walsh *et al.*, 2001).

In recent decades, antimicrobial resistance particularly multi-resistance has been considered as a public health problem worldwide. Vancomycin resistant bacteria, which are of major medical concern, have emerged in the first place due to the misuse of vancomycin to treat Gram-positive bacteria (Geornaras and Holy, 2001). Such a problem can have devastating effects on the efficacy of antimicrobials in treating human food-borne diseases. The excessive and improper usage of antimicrobial agents could be responsible for the emergence of resistant bacteria. Although, the use of antimicrobials in poultry production phase has reduced the risk of infectious disease, it may lead to dissemination of antimicrobial-resistant bacteria including resistant strains of *Listeria* in the environment.

The transmission of the resistant strains to human via contaminated food products may have public health consequences (Filiouisis *et al.*, 2009).

High prevalence of multidrug resistance indicates serious need for antibiotics surveillance programme and control of antibiotics sale and usage. Multiple antibiotics resistance (MAR) index of the *Listeria* isolates showed that the mean MAR index was 0.75 ± 0.066 . According to Wong *et al.*, (2012), MAR index of greater than 0.2 indicates that the isolates originate from high risk area where the rate of antibiotics usage is high.

The *Listeria monocytogenes* isolates were found to harbour *hylA* gene, which is agreement with the result obtained by Jallewor *et al.*, (2007) who reported the carriage of *hylA* by almost all isolates of *Listeria monocytogenes*. The presence of *hylA* virulence gene in a multiple drug resistant bacteria is of great concern to clinicians in the treatment of patients infected by such (Ndahi, *et al.*, (2013). The fact the organism was isolated from chicken carcasses raises the possibility of cross-contamination of such an isolates in the kitchen with ready to eat food or during storage in the refrigerator with other ready to eat foods.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1

Conclusion

Chicken carcasses and poultry processing environment in Zaria, Nigeria are contaminated with *Listeria* species at a prevalence of 9.71%. The prevalence of *Listeria* species in dressed chicken carcasses was 8.11%, broiler and local chicken carcasses have prevalence values of 8.44% and 7.79 % respectively of *Listeria* species. The prevalence of *Listeria* species in poultry processing environment was 14.42%. These prevalence values are of public health concern, among the humans and animals because contaminated chicken carcasses can cross-contaminate other foods during preparation in the kitchen.

This study has also shown that isolates exhibit multi resistance to commonly used antimicrobial agents suggesting indiscriminate use of these antimicrobial agents for therapeutic purposes in veterinary medicine which may lead to the development of antibiotic resistance. The results also showed that gentamicin and ciprofloxacin are the drug of choice in the treatment of listeriosis because the 40 *Listeria* isolates were 85% and 70% sensitive to these drugs.

Detection of *hyl A* in *Listeria monocytogenes* isolates showed that isolates have a virulence potential and thus the possibility of causing listeriosis. Multi-drug resistance in virulent *Listeria* species is of serious concern to public health and clinician

6.2 Recommendations

- 1 Poultry farmers should be educated on the danger of indiscriminate usage of antimicrobial agents in safe guarding the health and wellbeing of their birds.

2. Chicken vendors or butchers should always ensure good sanitary conditions within and around the poultry processing environment and also maintain a good personal hygiene and disinfect working surfaces before and after slaughter processes.
3. Consumers should avoid storage of raw chicken carcasses (RCC) in the refrigerator with ready to eat foods (RTF) to limit the possibility of cross contamination between the RCC and RTF.
4. Further studies should involve other meat products such as beef, mutton, pork meat etc. to ascertain their level of contamination with *Listeria*.
5. Future researches should focus on other nosocomial bacterial infections which could transmit zoonotic pathogens to human population via poultry carcasses.
6. Field trials should involve other antibiotics such as quinolones, methicillin
7. Rampant public health education, enlightenment and awareness campaigns is needed in poultry processing plants.

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APPENDICES

Appendix I:

Chemicals, Media and Reagents Used for *Listeria* species isolation

Chemical Composition of Pre- Enrichment Broth

Listeria enrichment broth was used (LEB)

Specifications; KM 10505

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

Ingredients;

| | |
|-----------------|-------|
| Peptone mixture | 20 g |
| Yeast extracts | 6.0 g |

| | |
|--------------------------------|------------|
| Sodium chloride | 5.0 g |
| Potassium dihydrogen phosphate | 2.5 g |
| Glucose | 2.5 g |
| Nalidixic Acid | 0.04 g |
| Acriflavin HCL | 0.015 |
| Cyclohexamide | 0.05 |
| pH; | 7.3+/- 0.2 |

Preparation of Mueller Hinton Agar

36.1 gm of powder was weighed and added to 1 l000ml of deionized water (conductivity <10ms) and then warmed until complete dissolution. It was mixed well and 225 ml was distributed into each of 250ml erlenymer flasks and sterilized by autoclaving at 121 0C for 15'.

Chemical composition Mueller Hinton Agar (CM0337, Oxoid, Basingstoke, UK)

| | |
|--------------------------|---------|
| Beef dehydrated infusion | 300.00g |
| Casein Hydrolysate | 17.5g |
| Starch | 1.5g |
| Agar | 17.0g |

| | |
|-----------------|-----------------|
| Distilled water | 1000ml |
| pH | 7.3±0.1 at 250C |

Preparation

Suspend 38g of Mueller Hinton agar base in to 1000ml of water, heat gently to dissolve completely and sterilized by autoclaving at 121⁰C for 15minutes

Chemical composition of Nutrient Agar (CM0337, Oxoid, Basingstoke, UK)

| | |
|-------------------------|-----------------|
| Lab –lemco beef extract | 1.0g |
| Yeast extract | 2.0g |
| Peptone | 5.0g |
| Sodium Chloride | 5.0g |
| Agar | 15.0g |
| Distilled water | 1000ml |
| pH | 7.3±0.1 at 250C |

Medium preparation

Suspend 28g of the agar base into 1000ml of distilled water, heat gently to boil to dissolve completely and sterilized by autoclaving at 121⁰c for 15 minutes

Chemical composition of Andrade peptone water (CM0061, Oxoid, Basingstoke, UK)

| | |
|---------|-------|
| Peptone | 10.0g |
|---------|-------|

| | |
|---------------------|------------------------------|
| Sodium chloride | 5.0g |
| Andrade's indicator | 0.1g |
| pH | 7.3±0.1 at 25 ⁰ C |

Suspend 15g of the medium into 1000ml of distilled water.

Chemical Composition of Brain Heart Infusion

| | |
|-----------------------------|--------|
| Brain Heart infusion solids | 17.5g |
| Tryptose | 10.0g |
| Glucose | 2.0g |
| Sodium chloride | 5.0g |
| Disodium phosphate | 2.5g |
| Distilled water 1litre | 1000ml |

***Listeria* selective agar base (Oxford formulation)**

Formula per Litre

| | |
|--------------------------|-----------|
| Columbia blood agar base | 39g |
| Aesculin | 1.0g |
| Ferric ammonium citrate | 0.5g |
| Lithium Chloride | 15.0g |
| pH | 7.2 ± 0.2 |

Chemical Composition of *Listeria* Selective Supplement (Oxford Formulation SR140)

Vial Contents

SR 140E to supplement 500ml of *Listeria* agar medium

| | |
|-------------------|-------|
| Cycloheximide | 200mg |
| Colistin sulphate | 10mg |
| Acriflavine | 2.5mg |
| Cefotatan | 1.0mg |
| Fosfomycin | 5.0g |

Composition of Gram's Stain Reagents

| | |
|---------------------------|--------|
| Crystal Violet Gram Stain | 1000ml |
| Crystal violet | 20g |
| Ammonium oxalate | 9g |
| Ethanol (absolute) | 95ml |
| Distilled water | 905ml |
| Lugol's Iodine Solution | 1000ml |
| Potassium iodide | 20g |
| Iodine | 10g |
| Distilled water | 1000ml |

Acetone-Alcohol Decolourizer (1000ml)

| | | |
|------------------------|----|--------|
| Acetone | | 500ml |
| Ethanol (absolute) | | 475ml |
| Distilled water | | 25ml |
| Neutral Red (0.1% w/v) | | 1000ml |
| Neutral red | 1g | |
| Distilled water | | 1000ml |

Composition of Sheep Blood Agar for Beta and alpha Haemolysis

| | | |
|--------------------------|--|--------|
| Blood agar base (Merck®) | | 40g |
| Sheep blood 7% (1.4×50) | | 70ml |
| Distilled water | | 1000ml |

Composition of Nutrient Agar for Catalase Test

| | | |
|--------------------------|--|--------|
| Blood agar base (Merck®) | | 40g |
| Distilled water | | 1000ml |

Chemical Composition of Motility Test Medium

| | | |
|------------------------------|--|----|
| Motility test medium (Difco) | | 8g |
| Nutrient Broth (Difco) | | 2g |

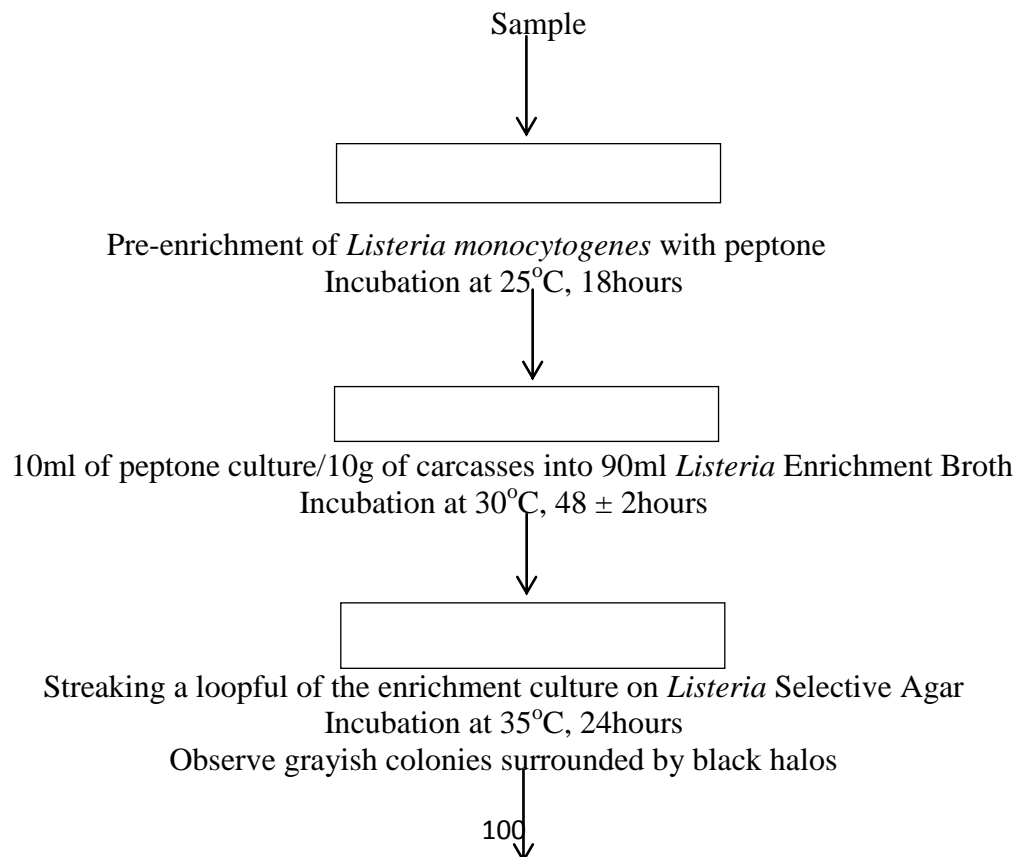
| | |
|-----------------|-------|
| Sodium Chloride | 0.5g |
| Distilled water | 500ml |

Chemical Composition of Carbohydrate (Sugar) Fermentation (Difco C-836-01)

| | |
|---------------------|------|
| Peptone | 10g |
| Meat extract | 3g |
| Sodium chloride | 5g |
| Andrade=s indicator | 10ml |
| Distilled water | 100 |

Appendix II:

Flow Chart for the isolation and identification of *Listeria* species



[]

Select 5 presumptive colonies for confirmation

If well separated colonies are available, streak one colony on Tryptone soya yeast extract agar

agar



[]

Gram staining, Motility, β -hemolysis



[]

β -Haemolysis, Carbohydrate fermentation/ Acid production



[]



[]



[]

Appendix III:

Chemicals and Reagents for gene amplification

Chemical Composition of Buffer for PCR Gene Amplification(R)

Standard buffer for PCR amplification with AmpliTaq or nature Taq DNA polymerase are the GeneAmp®.

GeneAmp 10 × PCR buffer is composed of 500nm Potassium chloride and 100nm Tris HCL (pH8.3 at room temperature), 15nm magnesium chloride and 0.01% (w/v) gelatin.

Dilution = for AmpliTaq DNA polymerase is 0.15%,

And NP - 40, 0.15% Tween(R) 20, 0.1nm EDTA, and 200µm (each) dGTP, dATP, dTTP and dCTP (10mM Tris-chloride [pH8.5], 1.15mM MgCl₂, 50 mM KCl) were mixed and kept at room temperature).

Chemical Composition of Agar Gel Buffer 1Litre (TBE)

Trisphosphate

108.9g

| | |
|-----------------|--------|
| Borate | 55.69g |
| EDTA | 9.3g |
| Distilled Water | 100ml |
| pH | 8.3 |

Chemical Composition of Buffer for Agarose Gel

| | |
|------------------|--------|
| Sucrose | 50 % |
| EDTA | 100µmg |
| Bromophenol blue | 0.1 % |

Done according to manufacturer's procedure.

Chemical Composition of 1.5%% Agarose Gel

Preparation:

1.5g of Agarose was dissolved in 100ml of 0.5 Triple buffered saline (TBS) and autoclaved in microwave oven. The gel was then poured onto an appropriate glass of 200mm by 200mm in diameter. This was allowed to cool to room temperature prior to usage.

Chemical Composition of Phosphate Buffered Saline

| | |
|-------------------------|------|
| Sodium Chloride | 8g |
| Disodium orthophosphate | 1.5g |
| Potassium Chloride | 0.2g |
| Distilled water | 1000 |

Chemical Composition of Enzyme Solutions for Lysing *Listeria* Lysozyme Solution 120mg/ml in Water

12mg of Lysozyme (Sigma) was weighed into Eppendorf tube containing 100ml of distilled water. This was allowed to dissolve and stored at -20°C.

Proteinase K Solution (12mg /ml in water)

1.2g (Sigma) was weighed into Eppendorf tube containing 100ml of distilled water. This was allowed to dissolve and stored at -20°C. 220

Chemical Composition of Agar Gel Buffer 1Litre (TBE)

| | |
|---------------|--------------|
| Trisphosphate | 108.9g |
| Borate | 55.69g |
| EDTA | 9.3g |
| Distilled | Water 1litre |
| pH | 8.3 |

Chemical Composition of Buffer for Agarose Gel

| | |
|------------------|-----------|
| Sucrose | 50 % |
| EDTA | 100µmg |
| Ethidium bromide | 0.1 % 221 |

Chemical Composition of 1.5% Agarose Gel

Preparation:

1.5g of Agarose was dissolved in 100ml of 0.5 Triple buffered saline (TBS) and autoclaved in microwave oven. The gel was then poured onto an appropriate glass of 200mm by 200mm in diametre. This was allowed to cool to room temperature prior to usage.

Chemical Composition of Phosphate Buffered Saline

| | |
|------------------------|------|
| Sodium Chloride | 8g |
| Disodiumorthophosphate | 1.5g |
| Potassium Chloride | 0.2g |

Distilled water

1,000ml

Appendix IV:

Chemical Composition of 1.5% Agarose Gel

Preparation:

1.5g of Agarose was dissolved in 100ml of 0.5 Triple buffered saline (TBS) and autoclaved in microwave oven. The gel was then poured onto an appropriate glass of 200mm by 200mm in diameter. This was allowed to cool to room temperature prior to usage.

Chemical Composition of Phosphate Buffered Saline

| | |
|------------------------|---------|
| Sodium Chloride | 8g |
| Disodiumorthophosphate | 1.5g |
| Potassium Chloride | 0.2g |
| Distilled water | 1,000ml |

A plate showing a clear of inhibition on Muller Hinton Agar

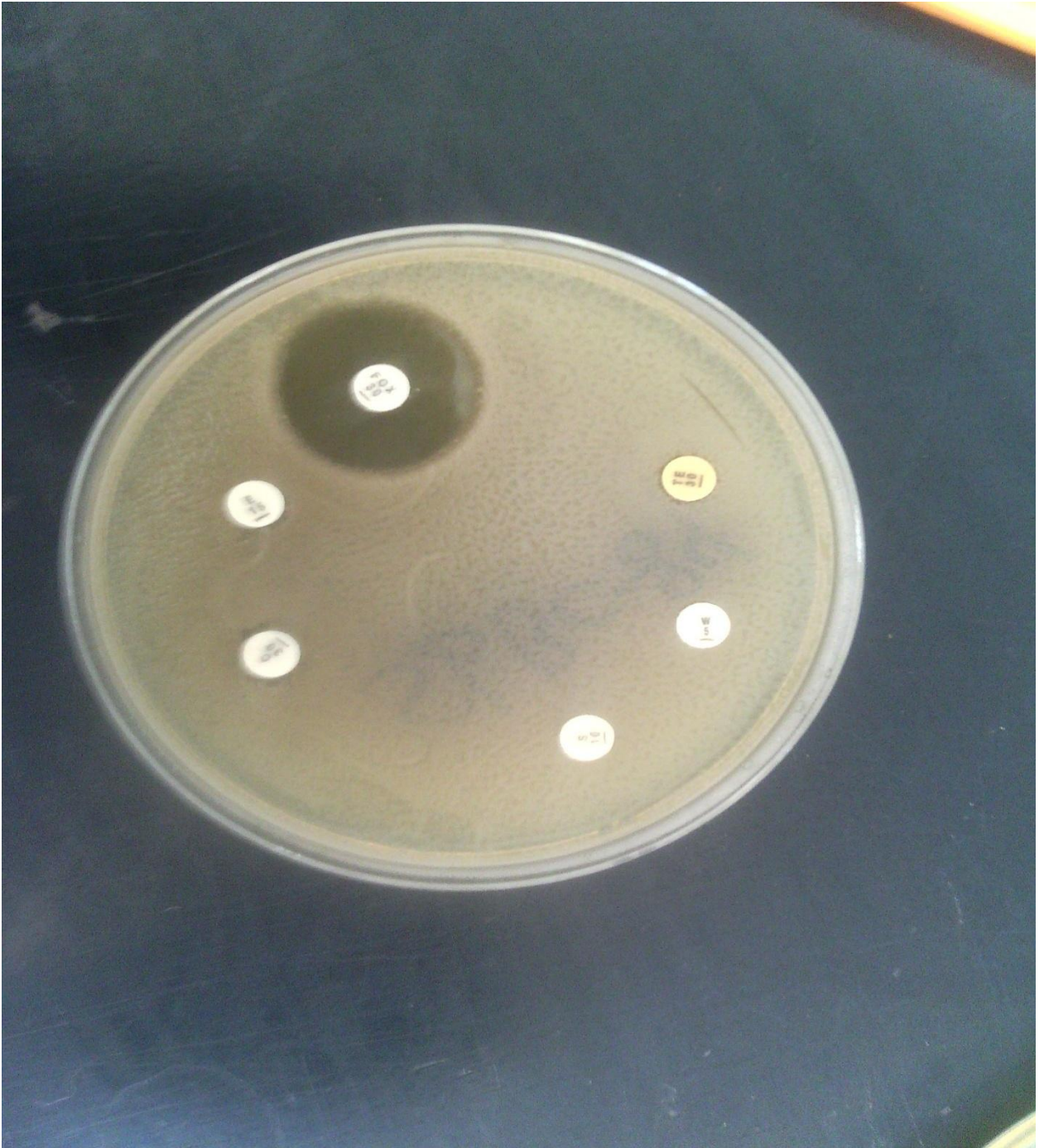


Plate III: A clear zone of inhibition on Muller- Hinton agar by one the antibiotic discs

Appendix V:

Zone diameter of antimicrobial agents according to CLSI guidelines 2011 using *Staphylococcus aureus* (ATCC 25923) as positive control

Zone diameter are in mm

| Antimicrobial | Symbol | Disk | Susceptible | Intermediatel | Resistant |
|-------------------|--------|--------------|-------------|---------------|-----------|
| Agent | | Content (µg) | | Susceptible | |
| Amoxicillin | AML | 10 | ≥20 | - | ≤19 |
| Chloramphenicol | C | 30 | ≥18 | 13-17 | ≤12 |
| Cefoxitin | FOX | 30 | ≥22 | - | ≤21 |
| Ciprofloxacin | CIP | 5 | ≥21 | 16-20 | ≤15 |
| Erythromycin | E | 15 | ≥23 | 14-22 | ≤13 |
| Gentamicin | CN | 10 | ≥15 | 13-14 | ≤12 |
| Nitrofurantoin | F | 30 | ≥17 | 15-16 | ≤14 |
| Tetracycline | TE | 15 | ≥19 | 15-18 | ≤14 |
| Trimethoprim | W | 5 | ≥16 | 11-15 | ≤16 |
| Sulfamethoxazole- | SXT | 25 | ≥16 | 11-15 | ≤16 |
| Trimethoprim | | | | | |
| Vancomycin | VA | 30 | - | - | - |

Appendix VI

Microgen™ *Listeria*-ID System for Identification and Confirmation of *Listeria* Species

| Sample number code | <i>Listeria</i> species | Percentage Probability (%) | Octal |
|-----------------------|-------------------------|-------------------------------|-------|
| 1. DLC 59 | <i>L. ivanovii</i> | 98.11 | 4261 |
| 2. SMLC 139 | <i>L. grayi</i> | 96.39 | 0200 |
| 3. SMLC 149 | <i>L. ivanovii</i> | 99.75 | 5200 |
| 4. SMB 94 | <i>L. ivanovii</i> | 96.25 | 4001 |
| 5. TB78 | <i>L. grayi</i> | 99.39 | 4240 |
| 6. SBBC 108 | <i>L. ivanovii</i> | 96.25 | 4001 |
| 7. SBLC 88 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 8. DLC 49 | <i>L. welshimeri</i> | 97.92 | 4020 |
| 9. SBBC 116 | <i>L. monocytogenes</i> | 96.29 | 4115 |
| 10. SBF 67 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 11. TB 77 | <i>L. grayi</i> | 99.39 | 4240 |
| 12. DB 84 | <i>L. ivanovii</i> | 99.7 | 4201 |
| 13. SBF 61 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 14. DB 82 | <i>L. ivanovii</i> | 97.63 | 0400 |
| 15. SMLC 155 | <i>L. ivanovii</i> | 98.71 | 5240 |
| 16. TF 59 | <i>L. ivanovii</i> | 96.25 | 4001 |
| 17. SBLC 83 | <i>L. ivanovii</i> | 99.7 | 4201 |
| 18. SMF 74 | <i>L. ivanovii</i> | 96.25 | 4000 |
| 19. DB 82 | <i>L. ivanovii</i> | 96.25 | 4000 |
| 20. SBBC 84 | <i>L. ivanovii</i> | 96.29 | 4020 |

| | | | |
|--------------|-------------------------|-------|------|
| 21. SMBC 128 | <i>L. ivanovii</i> | 96.25 | 4001 |
| 22. DLC 47 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 23. SMLC 122 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 24. SBBC 94 | <i>L. ivanovii</i> | 96.29 | 4001 |
| 25. DF 57 | <i>L. grayi</i> | 42.16 | 4002 |
| 26. SBLC 85 | <i>L. ivanovii</i> | 96.25 | 4001 |
| 27. SBBC 88 | <i>L. ivanovii</i> | 96.29 | 0000 |
| 28. DB 85 | <i>L. welshimeri</i> | 99.98 | 4060 |
| 29. TK 6 | <i>L. ivanovii</i> | 96.25 | 4001 |
| 30. DB 83 | <i>L. ivanovii</i> | 96.25 | 4001 |
| 31. SBBC 80 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 32. DBC 47 | <i>L. monocytogenes</i> | 96.25 | 4125 |
| 33. SBLC 112 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 34. SBBC 87 | <i>L. ivanovii</i> | 96.25 | 4002 |
| 35. SBBC 91 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 36. DT 33 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 37. SMBC 120 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 38. SMBC 132 | <i>L. ivanovii</i> | 96.29 | 4000 |
| 39. SMBC 131 | <i>L. ivanovii</i> | 96.29 | 4002 |
| 40. SBLC 84 | <i>L. ivanovii</i> | 97.29 | 4002 |

Appendix VII

Table 2.4: **Guide for biochemical identification of *Listeria* species**

| Species | Haemolysis | CAMP | | Sugar fermentation | | |
|-------------------------|------------|----------|---------|--------------------|----------|----------|
| | | S.aureus | R. equi | Xylose | Rhamnose | Mannitol |
| <i>L. monocytogenes</i> | + | + | - | - | + | - |
| <i>L. ivanovii</i> | - | - | + | + | - | - |
| <i>L. seeligeri</i> | + | + | - | + | - | - |
| <i>L. innocua</i> | +/- | - | - | - | V | - |
| <i>L. grayi</i> | - | - | - | - | V | + |
| <i>L. welshimeri</i> | - | - | - | + | V | - |

+ : positive

+/- : weakly positive

- : negative

CAMP: is an acronym for Christie, Atkins, Munch, Petersen; the discoverers of this haemolysis phenomenon

V: Variable

Adopted from Jemmi and Stephan (2006)

Appendix VIII:

Identification of *Listeria species* based pre and enrichment media and conventional biochemical test

| Sample spp. No. | Catalase | Rhamnose | Xylose | Mannitol | Oxidase | Aesculin | <i>Listeria</i> |
|-----------------|----------|----------|--------|----------|---------|----------|-----------------|
| TBC1 | + | - | + | + | - | - | |
| TBC2 | - | - | - | - | - | - | |
| TBC3 | + | - | + | + | - | + | |
| TBC4 | + | - | + | + | - | - | |
| TBC5 | - | - | - | - | - | - | |
| TBC6 | + | - | + | + | + | - | |
| TBC7 | + | + | + | + | - | + | |
| TBC8 | - | - | - | - | - | - | |
| TBC9 | - | - | - | - | - | - | |
| TBC10 | + | + | + | + | - | + | |
| TBC11 | + | + | + | - | + | + | |
| TBC12 | + | - | + | + | - | + | |
| TBC13 | + | - | + | + | + | + | |
| TBC14 | + | - | + | + | - | + | |
| TBC15 | - | - | - | - | - | - | |
| TBC16 | - | - | - | - | - | - | |
| TBC17 | - | - | - | - | - | - | |
| TBC18 | - | - | - | - | - | - | |
| TBC19 | - | - | - | - | - | - | |
| TBC20 | - | - | - | - | - | - | |
| TBC21 | + | - | + | + | + | + | |
| TBC22 | - | - | - | - | - | - | |
| TBC23 | - | - | - | - | - | - | |
| TBC24 | - | - | - | - | - | - | |
| TBC25 | - | - | - | - | - | - | |
| TBC26 | - | - | - | - | - | - | |
| TBC27 | - | - | - | - | - | - | |
| TBC28 | - | - | - | - | - | - | |
| TBC29 | - | - | - | - | - | - | |
| TBC30 | + | - | + | + | + | - | |
| TBC31 | - | - | - | - | - | - | |
| TBC32 | - | - | - | - | - | - | |
| TBC33 | - | - | - | - | - | - | |
| TBC34 | - | - | - | - | - | - | |
| TBC35 | + | - | - | + | + | - | |
| TBC36 | - | - | - | - | - | - | |

| | | | | | | |
|--------|---|---|---|---|---|---|
| TBC37 | + | - | + | - | - | + |
| TBC38 | - | - | - | - | - | - |
| TBC39 | + | + | + | + | + | + |
| DBC40 | - | - | - | - | - | - |
| DBC41 | + | - | + | + | - | - |
| DBC42 | - | - | - | - | - | - |
| DBC43 | - | - | - | - | - | - |
| DBC44 | - | - | - | - | - | - |
| DBC45 | - | - | - | - | - | - |
| DBC46 | - | - | - | - | - | - |
| DBC47 | + | + | - | - | + | + |
| DBC48 | - | - | - | - | - | - |
| DBC49 | - | - | - | - | - | - |
| DBC50 | - | - | - | - | - | - |
| DBC51 | + | + | + | - | - | + |
| DBC52 | - | - | - | - | - | - |
| DBC53 | - | - | - | - | - | - |
| DBC54 | + | + | + | - | + | + |
| DBC55 | - | - | - | - | - | - |
| DBC56 | - | - | - | - | - | - |
| DBC57 | - | - | - | - | - | - |
| DBC58 | - | - | - | - | - | - |
| DBC59 | - | - | - | - | - | - |
| DBC60 | - | - | - | - | - | - |
| DBC61 | - | - | - | - | - | - |
| DBC62 | + | - | + | + | + | + |
| DBC63 | + | - | + | - | - | + |
| DBC64 | - | - | - | - | - | - |
| DBC65 | - | - | - | - | - | - |
| DBC66 | - | - | - | - | - | - |
| DBC67 | - | - | - | - | - | - |
| DBC68 | + | - | + | + | + | + |
| DBC69 | - | - | - | - | - | - |
| DBC70 | + | - | + | + | + | - |
| DBC71 | + | - | + | + | + | + |
| DBC72 | - | - | - | - | - | - |
| DBC73 | - | - | - | - | - | - |
| DBC74 | - | - | - | - | - | - |
| DBC75 | - | - | - | - | - | - |
| DBC76 | - | - | - | - | - | - |
| DBC77 | - | - | - | - | - | - |
| DBC78 | - | - | - | - | - | - |
| SBBC79 | - | - | - | - | - | - |
| SBBC80 | + | - | + | - | - | + |
| SBBC81 | + | - | + | + | + | + |
| SBBC82 | + | + | + | + | - | + |
| SBBC83 | + | + | + | - | - | + |

| | | | | | | |
|---------|---|---|---|---|---|---|
| SBBC84 | + | - | + | + | + | + |
| SBBC85 | + | - | + | + | - | - |
| SBBC86 | + | + | + | - | - | + |
| SBBC87 | + | + | - | + | - | + |
| SBBC88 | + | - | - | + | + | + |
| SBBC89 | + | - | - | + | - | + |
| SBBC90 | + | - | + | + | - | + |
| SBBC91 | + | - | + | - | - | + |
| SBBC92 | + | - | - | + | - | + |
| SBBC93 | - | - | - | - | - | - |
| SBBC94 | + | - | - | + | + | + |
| SBBC95 | + | - | + | - | + | + |
| SBBC96 | - | - | - | - | - | - |
| SBBC97 | - | - | - | - | - | - |
| SBBC98 | - | - | - | - | - | - |
| SBBC99 | - | - | - | - | - | - |
| SBBC100 | - | - | - | - | - | - |
| SBBC101 | + | - | + | + | + | + |
| SBBC102 | - | - | - | - | - | - |
| SBBC103 | + | + | + | - | - | + |
| SBBC104 | - | - | - | - | - | - |
| SBBC105 | - | - | - | - | - | - |
| SBBC106 | - | - | - | - | - | - |
| SBBC107 | - | - | - | - | - | - |
| SBBC108 | + | - | - | + | + | + |
| SBBC109 | - | - | - | - | - | - |
| SBBC110 | - | - | - | - | - | - |
| SBBC111 | - | - | - | - | - | - |
| SBBC112 | - | - | - | - | - | - |
| SBBC113 | - | - | - | - | - | - |
| SBBC114 | + | + | + | - | - | + |
| SBBC115 | - | - | - | - | - | - |
| SBBC116 | + | + | - | - | + | + |
| SBBC117 | - | - | - | - | - | - |
| SMBC118 | + | + | + | - | - | + |
| SMBC119 | - | - | - | - | - | - |
| SMBC120 | + | + | - | + | + | + |
| SMBC121 | + | + | - | + | - | + |
| SMBC122 | + | - | + | + | + | + |
| SMBC123 | + | - | + | - | - | + |
| SMBC124 | - | - | - | - | - | - |
| SMBC125 | + | - | + | - | - | + |
| SMBC126 | + | - | + | + | + | + |
| SMBC127 | + | + | - | + | + | + |
| SMBC128 | + | - | + | - | - | + |
| SMBC129 | - | - | - | - | - | - |
| SMBC130 | - | - | - | - | - | - |

SMBC131 + + + - + +