STUDIES OF *LISTERIA MONOCYTOGENES* (BACTERIUM) IN SOME SELECTED LOCAL GOVERNMENT AREAS OF PLATEAU STATE, NIGERIA

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AUGUST, 2007

DECLARATION

I hereby declare that his work is the poduct of my own research efforts; undertaken under the supervision of Professor C.I.C. Ogbonna and has not been presented elsewhere for the award of a degree or certificate. All sources of information have been duly and appropriately acknowledged.

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CERTIFICATION

This is to certify that the research work

and the subsequent preparation of this project Report by

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ABSTRACT

Studies were carried out on the distribution, characterization, pathogenicity and antibiotic susceptibility of *Listeria monocytogenes* in Six Local Government Areas of Plateau State, Nigeria. A total of One hundred and fifty (150) experimental samples were taken from each test Local Government Area. The isolation was carried out with the aid of cold enrichment, selective broth and selective *Listeria* agar. The experimental samples examined included: cow, goat, poultry, rabbit and sheep wastes (faecal samples). The other samples investigated included: cultivated soil particles, farm wastes, human wastes (faeces), decaying leaves and water. A total of nine hundred samples were examined in all. Out of these, 189 (21%) were found to contain *Listeria* species which was found to be highly significant at 5% and 10% level of probability (P = 0.01; P = 0.05). The *Listeria* isolates included *L. monocytogenes* (41.3%), *L. ivanovii* (20.1%), *L. grayi* (14.3%), *L. welshimeri* (8.4%), *L. innocua* (6.9%), *L. murrayi* (5.8%) and *L. seeligeri* (3.2%).The molecular characterization of the various *L. monocytogenes* isolates through polymerase chain reaction (PCR) technique into serotypes, revealed that they belonged to 13 serotypes (serovars). *Listeria* species occurrences were found to be highest in rabbit wastes (33.3%), followed by sheep wastes (27.8%), farm debris (23.3%), soil samples (22.4%), cow waste (22.2%), poultry droppings (20.0%), leaves (17.8%) and then water (13.3%). Samples of Human wastes were found to contain the least percentage of *Listeria* species (11.1%). It was observed that temperature, pH range and moisture content affected the colonization of the experimental samples by *Listeria*. The polymorphic nature but peculiar morphological characteristics of *Listeria monocytogenes* were helpful in the identification of the organism. The other species of microorganisms isolated along with *Listeria monocytogenes* during the studies included; *Bacillus species, Brochothrix* species, *Kurthia* species, *Staphylococcus aureus, Streptococcus* species, *Candida albicans, Candida* species, *Aspergillus* species, *Mucor* species and *Penicilium* species. Oxford *Listeria* agar was observed to favour the isolation and molecular characterization of the *Listeria* species. The data generated from the various experimental samples were subjected to statistical analyses. The results showed that the occurrence of *L. monocytogenes* and other *Listeria* species in the experimental specimens was significant at 5% and 10% level of probability (P = 0.01; P = 0.05). The results also showed that the employment of different culture media for the isolation of *L. monocytogenes* and other *Listeria* species was significant at 5% and 10% level of probability (P = 0.01; P = 0.05). The results of the pathogenicity study of *L. monocytogenes* 4b isolate in immunocompromised albino mice revealed that the organism could infect mice which could subsequently lead to their death. The necropsy examinations of visceral organs of such infected mice, revealed gross pathological and diagnostic features that are known to be peculiar to listerial infection. The *L. monocytogenes* 4b isolate was sensitive *in-vitro* to Gentamycin, followed by Ampicillin and then Penicillin. Others were Enrofloxacin followed by Neomycin, Oxytetracycline and then Keproceryl. The *L. monocytogenes* 4b organism was resistant to Pefloxacin only. The results obtained from the studies would serve as a valuable resource baseline for listeriosis surveillance in the study areas in particular and Nigeria in general. The public health implications of the various results obtained have also been discussed.

CHAPTER ONE

INTRODUCTION

Despite the remarkable advances in medical research and treatments during the 20th century, infectious disease remains among the leading causes of death worldwide for three reasons: Emergence of new infectious pathogens; re-emergence of old infectious pathogens and persistence of intractable infectious pathogens (WHO, 1984). These have revealed a number of previously unknown human and animal pathogens such as *Listeria monocytogenes* (WHO, 1988; Gillespie *et al.*, 2006).

Listeria monocytogenes has been reported as the most heat resistant non-spore forming foodborne pathogen (Brown, 1991). It is the agent of listeriosis, a serious zoonotic infection caused by eating foods contaminated with *L. monocytogenes. L. monocytogenes* infects tens of millions of people every year in tropical and temperate countries, causing flulike symptoms and even death (Schuchat *et al.*, 1991a).

The disease can manifest itself in several ways, but the three principal ones are abortion, septicemia and meningitis or meningoencephalitis in humans with fatality rate, greater than 20 – 25% and animals with case fatality rate of 30 – 60% respectively (WHO, 1987; WHO, 1988; Gellin and Broome, 1989; Schuchat *et al.*, 1991a).

The organism has the propensity to invade the foetoplacental unit leading to the infection of the foetus. This often results in the expulsion of the foetus (abortion), in live birth of a congenitally infected neonate (Gellin and Broome,

1989; Schuchat *et al*., 1991a). As an intracellular pathogen, *L. monocytogenes* has the ability to invade non-professional phagocyte cells, where it survives and replicate (Goebel *et al*., 1991). This ability makes diagnosis and chemotherapy of infection very difficult and could in most cases result in treatment failures (Hof, 1991a).

It has *Listeria monocytogenes* is ubiquitous in nature and has been isolated from the normal intestinal microflora of many vertebrates and some inveterate species as well as water and vegetation (Gray and Killinger, 1966; Welshimer, 1968). *Listeria monocytogenes* has also been isolated from soil particles, silage and agricultural environments (Weis and Seeligeri 1975). Wild bird droppings have been implicated as the source of *L. monocytogenes* contamination of agricultural land (Fenlon 1985; Fenlon, 1986).

This organism also grows in a wide variety of conditions such as high salt, acid environment, freezing and refrigerator temperatures (Juntilla *et al*., 1988). In recent times, listeriosis has been recognized as a major important source of zoonotic disease of public health (WHO, 1988). Sporadic cases of mastitis in dairy cattle have also been associated with *L. monocytogenes.* The organism could be disseminated through dairy products and in view of this it poses a public health concern to the unsuspecting human consumers of such products. Humans usually get infected by this organism after the ingestion of food and food products that had earlier been contaminated with *L. monocytogenes* (Donnelly and Baigent, 1986; Donnelly *et al.,* 1987). Such infection is common in immunnocompromised persons and the old (Pinner *et al*., 1992).

Knowledge of the ecological distribution of *L. monocytogenes* amongst the livestock, poultry and agricultural environment in Nigeria is lacking. The present study, therefore, would establish the occurrence of *L. monocytogenes* in some Nigerian ecological sites. The study in particular would seek to establish the most predominant Serovar(s) (serotypes) of *L. monocytogenes* in some Nigerian livestock wastes and also in soil, vegetation and aquatic environments.

1.1 JUSTIFICATION OF THE STUDY

Bacterial pathogens that are associated with human food poisoning may originate from the farm, vegetation, soil or animal food products. Reports of *Listeria* infections in Africa are scanty and particulary in Nigeria, despite many pathonogonomic signs and symptoms suggestive of listeriosis. In Nigeria, it is common to find farmers and clinicians diagnosing listeriosis in the field and instituting treatment without confirmatory diagnoses.

Significant human demographics, behaviour and land use changes have been witnessed in Nigeria within the last decade. These changes have influenced the lifestyle of the citizenry contributing to new disease emergence by changing transmission dynamics to bring livestock and people into closer and more frequent contact with pathogens. Such changes have led to increases in human agricultural activities in both residential areas and their environments. Run-off water from such pathogenic infected agricultural wastes or soils and contaminated human wastes used for agricultural land fertilization could aid in the contamination of surface or ground water sources for domestic and drinking purposes.

The economic and social impact of *Listeria monocytogenes* on livestock and the Nigerian population are largely unknown. This is because there is still a paucity of knowledge on the ecological distribution, molecular and serotypes of *L. monocytogenes* and other *Listeria* species are prsentlly lacking.

This study, therefore, would be expected to bring to the fore, the public health implications of the organisms in the area of study. It will also set the backdrop for further studies in order to identify some possible environmental sources of contamination by *L. monocytogenes* of agricultural and other food products in Plateau State. The Six Local Government Areas of the Northern Senatorial District of Plateau State are located within the zone of the state that has a large concentration of livestock, commercial dairy products and salad vegetable crops. The animal wastes of commercial farms of this area are used directly as bio-organic fertilizer of agricultural lands in the region.

The investigation was aimed at obtaining ecological distribution and spread of the organism in the study area. This included investigations on the occurrence of the organism in livestock (cattle, sheep and goats; rabbit and poultry) wastes, human wastes, and agricultural environments (soil, vegetation, farm debris) and aquatic environment in the six Local Government Areas of Plateau State.

The data obtained on occurrence, distribution, characterization and pathogenicity of the organisms will form the baseline for future studies and will provide regulatory agencies with valuable baseline information on the establishment of public health resource policies for the diagnosis, control and prevention of *L. monocytogenes* (listeriosis).

1.2 SCOPE OF STUDY

This study was carried out in six local government areas (Jos- North, Jos- South, Jos- East, Bassa, Riyom and Barkin Ladi) of Plateau State Nigeria (9°54'N 8°53'N). Plateau State lies in the middle belt region and is one of the states that make up the central zone of Nigeria.

Experimental samples were collected at random from fiteen locations/villages where Livestock, poultry, crop and vegetable farms existed for each of the six test local governments. These included agricultural soil samples, vegetation from agricultural lands, farm debris, water samples, livestock faeces (cow, goats, sheep and rabbits), poultry droppings and human faeces.

Each sample was replicated 15 times for a particular experimental sample substrate.Five (5g) of each of the ten (10) sample types was collected from each of the fifteen (15) villages (making 150 samples). These samples were collected with the aid of sterile receptacles (rubber gloves) and then placed in sterile plastic bags and then sealed with plastic tape.

Laboratory investigations will be focused on the colour, temperature, pH, moisture contents, solid residue and microbilogical analysis of experimental samples. However, the microbilogical analysis will be based ontheclassical *Listeria* isolation, morphological, physiological and biochemical tests (Gram's reaction, motility, carbohydrate fermentation, Catalase and CAMP test reactions). Also, molecular characterization of *Listeria* isolates using polymerase chain reaction (PCR) and virulence study on some *Listeria* isolates in laboratory animals (mice).

1.3 AIMS AND OBJECTIVES

Generally, the broad aim of this study was to determine the occurrence, distribution and characterization of *L. monocytogenes* and other *Listeria* species in in the aforementioned experimental samples from the study area. To actualize the this project objectively, concentrated, on the isolation, characterization and pathogenenicity of the organism using some specific steps for more complete structural elucidation *in vitro* and *in vivo*.

The specific objectives include:

1. The determination of the distribution of *L. monocytogenes* in livestock wastes, human wastes, agricultural soils and their vegetation.
2. The evaluation of the efficiency of three solid isolation media (Oxford, Palcam and Nalidixic Acid Sheep Blood Agar) in the isolation and growth of *L. monocytogenes*.
3. The assessment of phenotypic and molecular characteristics of Nigerian Field Isolates of *L. monocytogenes* and other *Listeria* species.
4. The identifictioan of the spectrum of Serovar(s) or serotypes of *L. monocytogenes* amongst Nigerian field isolates.
5. The determination of the virulence of *L. monocytogenes* in laboratory animals (Mice).
6. Antibiotic susceptibility (Antibiogram) on *L. monocytogenes* 4b used in the laboratory animals.

CHAPTER TWO

LITERATURE REVIEW

2.1 *LSTERIA MONOCYTOGENES*

The genus *Listeriae* unlike *Salmonellae*, *Yersinae*, *Shigellae* and other pathogenic organisms is not named after its discoverer. *Listeria*, which causes listeriosis, was discovered by Murray and his colleagues in 1926, after a careful investigation of an outbreak of the disease among laboratory animals in Cambridge. They called the organism *Bacterium monocytogenes*. Other generic names such as *Erysipelothrix and Listerella* were also suggested. *Listeria* was given by Pirie in 1927. This name was given in order to honour Lord Dr Lister who first observed antisepsis. Thereafter, the disease was known as listerellosis for many years until the present name Listeriosis came into general use.

The disease was first reported in sheep amongst the large animals in New Zealand and Western Australia in 1925 while in humans the first report was in 1929 (Gill, 1925 and Nyfeldt, 1929). However, Seeliger (1990) reported that listeriosis may have occurred before the advent of Murray *et al.,* (1926) and were discussed under names like pseudotuberculosis and argentophilic septicaemia which were completely unknown in laboratory animals.

The genus *Listeria* could best be characterized into seven species. The genus is closely related to the genera *Brochothrix* and *Bacillus* (Collin *et al.,* 1991). The seven species are considered to represent two closely related, but distinct lines of descent, with *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri* and *L. welshimeri* forming one group known as true *Listeria* organisms

with *L. grayi* and *L. murrayi* representing the other line (Collins *et al.,* 1991; Jones, 1992). However, only *L. monocytogenes* and *L. ivanovii* are pathogenic for man and animals. Unlike *L. monocytogenes* which causes listeriosis in humans and animals, *L. ivanovii* is exclusively an animal pathogen and accounts for about 10% *Listeria* infections in animals (Jones, 1992).

The organism could be rod- shaped, coccoid or filamentous. The shape depends on nutrients, environmental and cultural conditions (Hass and Kreft, 1988). Among pathogenic bacteria, there are three distinctive properties that are peculiar to the genus *Listeria* which are of interest. These properties include the ability of the genus to survive and grow on within or very broad temperature range (0°C – 44°C), wide pH range (5.0 – 9.5), its ability to tolerate sodium chloride concentration of 5 – 25% and a variety of other toxic chemical such as tellurite, acridine dyes, lithium chloride, Nalidixic acid and cycloheximide. On blood agar plates, pathogenic *Listeria* exhibits zones of haemolysis indicating destruction of the erythrocytes by the toxic products of the organism. More so, it has the ability to survive in cells of the host's immune system or macrophages (Hass and Kreft, 1988; Schuchat *et al.,* 1991b).

2.2 THE DISTRIBUTION OF *LISTERIA* SPECIES

Listeria monocytogenes and other *Listeria* species are highly adaptable asporogenous motile organisms. Nevertheless, the ecological niche of *L. monocytogenes* is difficult to define. The organism is found in the soil, vegetation, plants, mud, water, sludge, hospital dusts and in the intestinal tract of man, animals and birds (Gray and Killinger, 1966; Weis and Seeliger 1975;

Fenlon, 1986). Their widel distribution in the environment may probably be due to their ability to grow at 4°C. This feature makes them very difficult to eliminate from food and dairy industries (Griffiths, 1989; Schuchat *et al.,* 1991).

The listerial biofilms formation and attachment on objects or substrates involves factors such as motility, specific attachment structures, long- and –short range attraction forces, hydrophobic forces and polysaccharides production (Marshall, 1986). Swaminathan and Feng (1994) reported that *L. monocytogenes* could radically alter its metabolism in order to utilize complex substrates by converting them into useable metabolites with the aid of bio emulsifiers in their biofilm colonies. These bioemulsifiers assist the organism in attaching themselves on surfaces in order to survive in nutrient – poor and otherwise hostile environments.

The attachment of the bacterium *L. monocytogenes* to a solid substrate is usually followed by microcolony formation. This microcolony then surrounds itself with extra-cellular polysaccharides (a glycocalyx) (Sutherland, 1980). The glycocalyx, besides being a physical barrier, is strongly anionic and serves as protection to the microcolony. Those microcolonies of other *Listeria* species ultimately become a firmly attached biofilm (Sutherland, 1980; Marshall, 1988; Constertion, 1987).

The biofilm formation or surface attachment of *L. monocytogenes* and other *Listeria* species has been investigated. Archer (1990) reported that attachment and microcolony formation or biofilm formation by *L. monocytogenes could* be very difficult to eradicate when formed. He further observed that studies

with biocidal agents conducted on any non biofilm formation could lead to false sense of security. Thus *Listeria* could disappear for a time after the application of a biocide, but will certainly reappear.

2.3 TAXONOMY AND GENERAL CHARACTERISTICS OF *LISTERIA* SPECIES

Taxonomically, *Listeriae* may be confused with other genera such as *Brochothrix*, *Erysipelothrix, Lactobacillus, Kurthia* and *Jonesia* (Jones, 1988). However, *Brochothrix* is non-motile and does not grow at 35°C but at 30°C. Also, *Erysipelothrix* is non-motile and catalase-negative. *Kurthia* is strictly an aerobe and does not produce acid from glucose. *Jonsia denitrificans* is Voges-Proskaüer - negative and reduces nitrate. *Lactobacillus* is largely non-motile; however, some strains are motile and catalase – negative with few exceptions (Jones, 1988).The details of these distinctions are presented in Table 1.

Table 1: Biochemical and Physiological differentiation of organisms closely related to *Listeria monocytogenes* and other *Listeria* species

Organisms	Motility	Temperature 35°C	Catalase	Strict aerobe	Facultative anaerobes	Acid from glucose	Voges-Proskaüe (V.P)
L. monocytogenes	+	+	+	+/-	+	+	+
Brochothrix species	-	-	+	-	+	+	+
Erysipelothrix species	-	+	-	-	+	+	v
Kurthia species	+	.	.	+	-	-	-
Lactobacillus species	-	.	+/-	.	.	.	.
Jonesia denitrificans	+	+	+	+	+	.	-

Jones (1988)

Key:

+ = Positve - = Negative

v= Variable . = Not applicable

2.4 PHYSIOCHEMICAL CHARACTERISTICS OF *LISTERIA* SPECIES

According to Seeliger and Jones (1986), *Listeria* species are short, regular rods of 0.4 – 0.5μm by 0.5 – 2.0μm with rounded ends. They could be curved, occurring singly or in short chains, often presenting a "V"–shape. *Listeria* species are non-acid fast; do not form capsules and spores. They are motile (tumbling) and have a few peritrichous flagella.

These flagella are best expressed at 2°C to 25°C and least at 37°C (Seeliger and Jones, 1986). They are aerobic and facultatively anaerobic with optimum temperature of 30°C-35°C and have growth limits of 1°C – 45°C (Farber and Peterkin, 1991). In old or rough cultures, filaments of about 6-20μm could develop with irregular staining reactions as against the usual Gram's positivity by young cultures of the organism (Gutenkunst and Pine, 1992).

L. monocytogenes and other *Listeria species* are catalase – positive, oxidase- negative, methyl red- positive, Voges-Proskaüer (VP) – positive and indole negative. They do not hydrolyse urea but utilize glucose and aesculin with the production of acid with no gas. The five true *Listeria* species do not utilize manitol or hydrolyse sodium hippurate. *L. grayi* and *L. murrayi* hydrolyse sodium hippurate and utilize manitol. On nitrate reduction, only *L. murrayi* reduces nitrate to nitrite (Weaver, 1989).

Prior to this period, *Listeriae* were placed in the family *Corynaebacteriaceae.* However, of recent, with the aid of molecular biology studies of the organism, *Listeriae* have been found to be are closely related to

Bacillus, *Lactobacillus* and *Streptococcus*. Thus by its rRNA sequence data, *Listeriae* are closest to *Brochothrix* and together with *Staphylococcus* and *Kurthia*, occupy a position between the *Bacillus* group and the *Lactobacillus*/*Streptococcus* group within *Clostridium – Lactobacillus – Bacillus* branch, where the mole percent guanine plus cytosine (G + C) of deoxyribonucleic acid (DNA) of all members is less than fifty (Jones, 1988; Collins *et al.,* 1991).

The recognized species of the genus *Listeria* includes *Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria welshimeri, Listeria innocua, isteria grayi* and *Listeria murrayi, Listeria denitrificans.* Apart from the aforementioned phylogenetic similarities, genetic transfers and immunological reactions often occur amongst *Listeria*, *Bacillus* and *Streptococcus* (Jones 1992).

Furthermore, Ludwig *et al. (*1984) had investigated the 16S rRNA of *L. monocytogenes* and *Brochothrix thermosphacta*, and reported that *Brochothrix* shares 338 common purine and pyrimidine bases with *L. monocytogenes*. They also observed that *Eryseipelothrix* even though in the *mycoplasma* group shared at least 23 oligonucleotides in common with *L. monocytogenes* and *Brochothrix*. *L. monocytogenes* contains teichoic and lipoteichoic acids similar to that of Bacilli *staphylococci*, *Streptococci* and *Lactobacilli* on the cell wall (Fiedler and Seger, 1983; Fiedler *et al*., 1984, Fiedler, 1988).

Despite the molecular relationship of *Listeria species* with other pathogens, there are also molecular differentiating characteristics within the seven recognized *Listeria species*. The *L. monocytogenes*, *L. ivanovii*, *L. seeligeri, L. welshimeri* and *L. innocua* are more closely related to each other than *L. grayi* and *L. murrayi*.

The mole percentage (G +C) of deoxyribonucleic acid (DNA) of the first five *Listeria* species ranges from 36 to 38, while the latter two species ranges from 41 to 42 percent (Collins *et al.*, 1991). The details of this are presented in Table **2**2.

Table 2: Some physiological and biochemical characteristics of *Listeria* species

Species	Xylose	Lactose	Galactose	Rhamnose	Manitol	Hippurate Hydrolysis	*Stphyloc occus aureus*	*Rhodoc occus equi*	β Haemolysis	Moles % G + C
Listeria monocytogenes	-	V	V	+	-	+	+	-	+	37-39
Listeria ivanovii	-	+	-	(+)	-	+	-	-	-	36-38
Listeria Seeligeri	+	-	-	-	-	-	+	-	W	36
Listeria welshimeri	+	-	-	V	-	-	-	-	-	36
Listeria innocua	+	+	V	-	-	+	-	+	++	37-38
Listeria grayi	-	+	+	-	+	-	-	-	-	41-42
Listeria murrayi	-	+	+	V	+	-	-	-	-	41-42.5

Notes: + = positive

W = weak

v = variable

Collins *et al.,* 1991)

Serologically, *L. grayi* and *L. murrayi* are distinct from the other five *Listeria* species. Thus, based on the degree of DNA – DNA hybridization, *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. innocua*, form a rather tight cluster and clearly belong to the same genus often reported as true *Listeria* species (Rocourt *et al*., 1983; Rocourt, 1988 and Collins *et al.,* 1991).

2.5 INTERGENERIC RELATIONSHIP OF *LISTERIA* SPECIES

All the phenotypic classification of the genus *Listeria* points to the facultatively anaerobic, catalase-negative genera *Lactobacillus*, *Streptococcus*, *Enterococcus* and *Lactococcus*; the facultatively anaerobic or aerobic, catalase-positive genera *Staphylococcus*, *Bacillus* and *Kurthia* and very close to the facultatively anaerobic, catalase positive genus *Brochothrix*. On the basis of 16S rRNA cataloguing studies and analysis of the 16S rRNA of bacteria of this group, all these genera contain DNA with a molecular percentage (G+C content) of less than 55 and are members of the *Clostridium*-*Lactobacillus*-*Bacillus* branch of the gram positive bacteria phylogeny (Stackebrandt and Woese 1981; Stackebrandt *et al*., 1983; Rocourt *et al*., 1987) The details of this are presented in Figure 1.

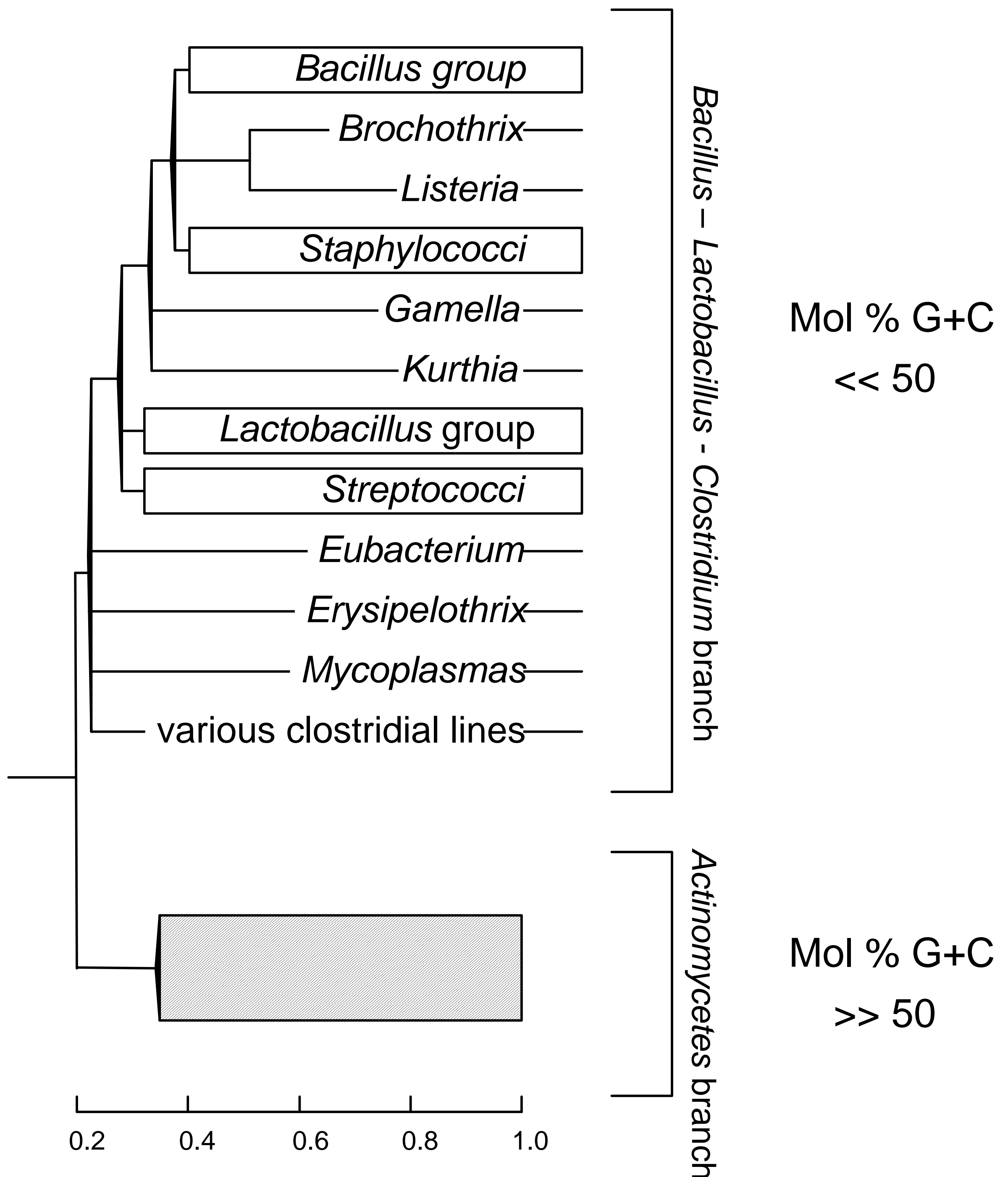

Figure 1: The relationships between *Listeria* and other Gram-positive bacteria based on 16S rRNA cataloguing (Stackebrant and Woese, 1981; Stackebrant *et al.*, 1983; Rocourt *et al.,* 1987)

Listeria places closest to *Brochothrix* from 16S rRNA sequence data on phylogenic position. These two genera, together with *Staphylococcus* and *Kurthia* occupy a position between *Bacillus* group and the *Lactobacillus*/*Streptococcus* group within the *Clostridium*-*Lactobacillus*-*Bacillus* branch (Rocourt *et al*., 1982; Kandler and Weiss, 1986). The details of this are presented in Figure 1. Further studies, employing the use of reverse transcriptase, revealed that from sequencing of 16S rRNA and phylogenetic interrelationships of *Listeria*e and other gram- positive taxa analysis the *Listeriae* occupy a position between *Brochothrix* and *Bacillus* in the phylogenic tree (Collins *et al.,* 1991), (Figure 2).

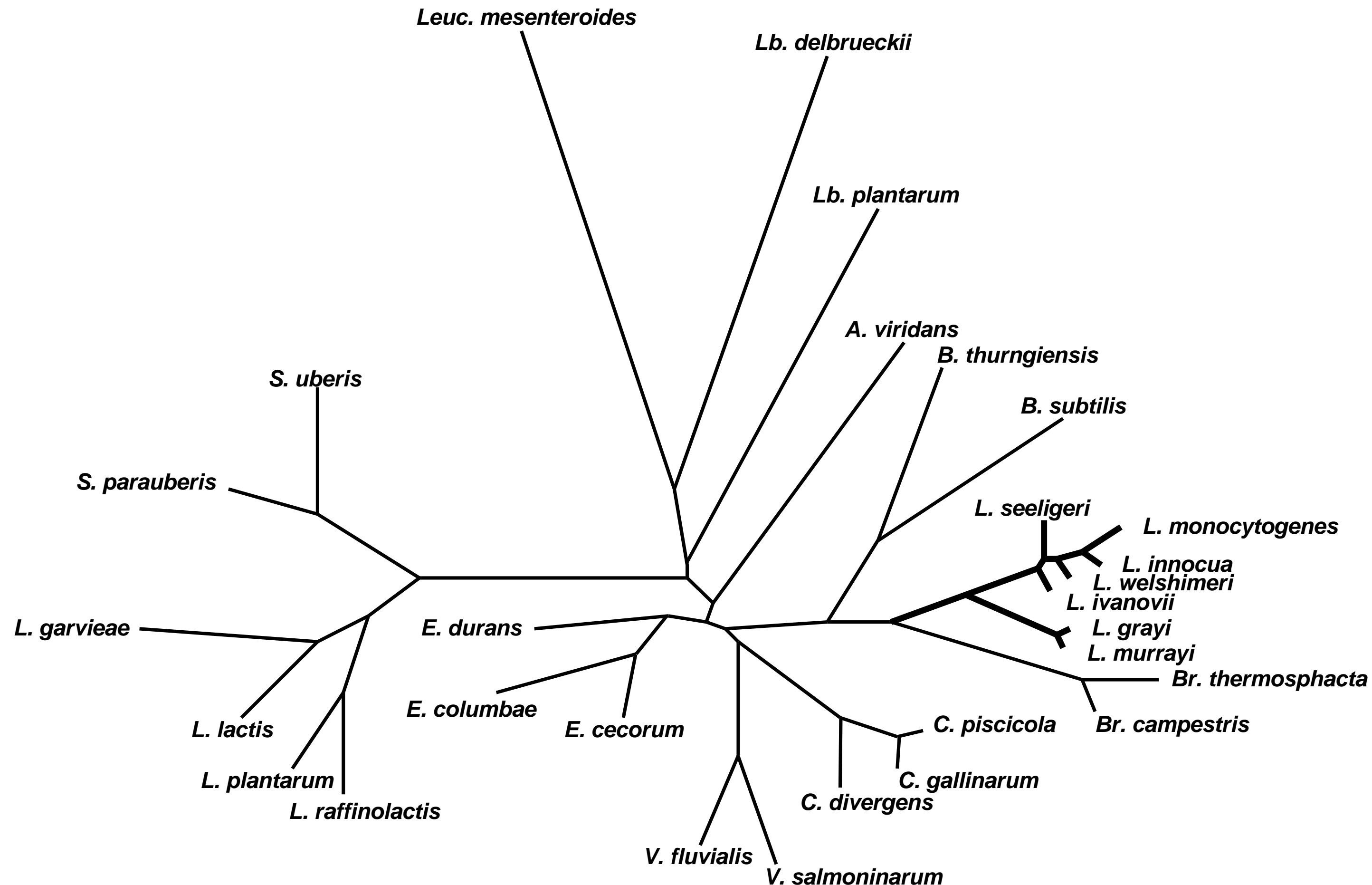

Figure 2: Unrooted tree or network showing the phylogenetic interrelationships of *Listeriae* and other low-G + C–content gram-positive taxa

The tree is based on a comparison of a continuous stretch of 1,340 nucleotides: the first and last bases in the sequence used to calculate K_{nuc} values correspond to positions 107(G) and 1433(A), respectively, in the *E. coli* sequence. Abbreviations: *A, Aerococcus; B, Bacillus; Br, Brochothrix; C, Carnobacterium; E, Enterococcus; L, Lactococcus; Lb, Lactobacillus; Leuc, Leuconostoc; List, Listeria; S, Streptococcus; V, Vagococcus* (Collins *et al*., 1991).

Stuart and Welshimer (1974) had earlier advocated for the placement of *L. grayi* and *L. murrayi* in the genus *Murraya* (as *M. grayi* subspecies *murrayi)*. However, by numerical taxonomic studies, Seeliger and Langer (1989) reported that *L. grayi* and *L. murrayi* share 81 to 87% similarity with the other five *Listeria* species with differences only on the flagella (H) antigens which do not cross-react.

On the cell wall composition, Fiedler *et al.,* (1984) reported that the poly ribitol-phosphate, type of teichoic acids are prevalent on the cell – wall polymer in *Listeria* species including *L. grayi* and *L. murrayi.* They further observed that lipoteichoic acids of *L. grayi* and *L. murrayi* are modified types. This also further separates the two species from the other five *Listeria* species. Studies on this modified lipoteichoic acids revealed that the modification may account for the *L. grayi* and *L. murrayi* resistance to the bacteriophages that lyse the five other *Listeria* species (Loessner and Busse, 1990).

2.6 PHYSIOLOGY AND METABOLISM IN *LISTERIA MONOCYTOGENES*

The physiology and metabolism of *L. monocytogenes* affect the aetiology and pathogenicity of the organism (Benedict, 1990). Such parameters are the facultatively anaerobic growth in the presence of respiratory enzymes and co-factors, catalase and super oxide dismutase which circumvent the macrophagic intracellular oxidative bursts. Others are the production of haemolysin phosphatase C, specific attachment to intestinal peyers patches and the crossing of placental and brain membrane barriers. These physiological activities, however, have relevance to the organism’s virulence and growth as well as infectivity.

Listeria monocytogenes ferments a number of sugars after 24 hours and somethimes delayed. It does not ferment some sugars and hydrolyses aesculin and salicin. The organism is also negative in indole production and utilization of citrate. The details are shown in Table 3.

Table 3: Some physiological and biochemical characteristics of *Listeria monocytogenes*

Reactants Carbohydrates and chemicals	ferments	rhamnose	glucose	fructose	mannose	galactose	cellubiose	trehalose	but not xylose	maltose	Sucrose	lactose	dextrin	polyhydric alcohols	glycerol	sorbitol	Hydrolysis of aesculin	Salicin	indole	citrate	starch	arabinose	inositol	mannitol
Listeria monocytogenes	+	+	+	+	+	+	+	+	-	D	D	D	D	D	D	D	+	D	-	-	-	-	-	-

Key:

+ = Positive

- = Negative

D = Delayed

(Pine *et al.*, 1988)

L. monocytogenes is partially inhibited by 0.02% azide and cyanide, and does not reduce nitrates to nitrite or produces hydrogen sulphide. On enzyme production, it is negative for urease, coagulase, arginine decarboxylase and the decarboxylases of glutaminase and arginine dihydrogenase. Moreso, the organism has no cytochromic C oxidase and is negative for tribietyrinase (Leighton *et al.*, 1975).

The biotypical and serological reactions of *L. monocytogenes* and other *Listeria* species like other organisms occur with substances at the cell-wall level. Based on cell wall composition, they are classified by the structure and composition as Gram-positive organisms, but are similar to Gram-negative pathogens in virulence characteristics (Fiedler, 1988). The major differences between the Gram-positive and Gram-negative bacteria are mainly the thickness and composition of the cell wall.

The Gram-negative organisms cell wall is about 2-10 nm thick, and contains an inner plasma membrane of proteins and lipid bi-layer which acts as an intervening periplasma space, a thin interwoven peptidoglycan, followed by an outer membrane. The outer membrane consists of protrutions from the plasma membrane and inclusions of flagella and amphipathic lipopolysaccharides and glycocalyx. Other amphipathic components of Gram-negative cell wall are phospholipids, proteins, glycolipids and lipoproteins (Fiedler, 1988).

In contrast to Gram-negative, Gram-positive cell wall has a thickness of 25-50nm and a much thicker interconnection of peptidoglycan layer. Within this

peptidoglycan layer are the external surfaces known as teichoic acids, defined as an alditol in a layer sequence, flanked by phosphodiesters. The alditol in *L. monocytogenes* is ribitol and various sugar molecules that may be attached to ribitol through the OH – linkage (Fiedler, 1988). *Listeria monocytogenes* has lipoteichnoic acids, from the inner lipid bilayer amphipathic components of *L. monocytogenes* which can further form micelles compounds with other cellular components of the organism or host cell (Fiedler, 1988).

These lipoteichnoic acids are the principal membrane to the surface.The peptiodoglycans, also known as murein or mucopeptides, are composed of glycan chains which are composed of alternating P 1-4 linked ad-acetylglucosamine and N – acetyl murramyl residues cross linked through the murramyl residues by an inter peptides linkage composed of alternating D and L amino acids (Fielder and Rhuland, 1987). In the case of *L. monocytogenes* and other *Listeria* species, there is diaminopimeic acid (DMA) in place of lysine found in many peptidoglycans. Similarly, the complex attachment of teichoic acids to the peptidoglycans is through the murramyl residues (Fiedler and Rhuland, 1987

2.6.1 Metabolism in *Listeria monocytogenes*

The metabolism of sugars by *L. monocytogenes* and other *Listeria* species for growth is homolactic. This is, however, dependent on the redox potential of the medium. Thus, aerobic or facultatively anaerobic nature of the growth medium influences the type of acid produced (Pine *et al.*, 1988). Lactic acid is the only product formed when grown anaerobically. When grown aerobically, Lactic acid and acetic acid or only acetic acid will be produced. Acetic acid accounts for

80-100% of products by sugar metabolized by *L. monocytogenes* and other *Listeria* species aerobically (Pine *et al.*, 1988).

Based on temperature, at 37°C glucose is fermented by the classical Embden – Meyehoff anaerobic glycolysis pathway to yield pyruvate and lactate with the production of up to 6mol ATP per mole of glucose (Atlas, 1984). The growth of *L. monocytogenes* and other *Listeria* species is proportional to the amount of sugar but dependent on the nature of the sugar being utilized. Studies by Pine (1988) indicated that *L. monocytogenes* with glucose as a substrate under aerobic conditions would have a generation time of approximately forty (40) to sixty (60) minutes and approximately 24 hours at 5°C.

Using the same substrate, the growth of *L. ivanovii* and *L. seeligeri* at 37°C is slower with a generation time of seventy-five (75) minutes and thus growth is delayed compared with *L. monocytogenes* at 5°C. He also reported that at aerobic and semi-anaerobic conditions, *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. grayi* and *L. murrayi* would exhibit excellent growth on a variety of common organically complex laboratory media, while *L. seeligeri* and *L. ivanovii* would show less growth (Pine *et al.*, 1988).

2.7 ISOLATION AND IDENTIFICATION OF *LISTERIA MONOCYTOGENES*

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 chemsul 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 thocyanate and potassium tellurate (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 asculin, 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, in 1988, Lovett modified this medium by the removal of the blood component. This modification also favoured isolation of *L. monocytognes* from foods.

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 – phenyethanol – 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 sort to develop 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).

2.8 CULTURE OF *LISTERIA MONOCYTOGENES*

Several investigations have compared the various selective broth as well as solid agar media for the isolation and identification of *L. monocytogenes* 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 *L. monocytogenes* in samples, the shortening of culture time period and detection of

heat injured or stressed organisms that may rejuvinate 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.

2.9 ENRICHMENT METHOD FOR THE ISOLATION OF *LISTERIA MONOCYTOGENES*

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's (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 Vermount 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 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).

2.9.1 Employment of Solid Media for the Isolation of *Listeria* species

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 and/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).

2.9.2 Confirmation Tests for *Listeria monocytogenes*

The final phase of the analysis for *L. monocytogenes* is the comfirmation 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-Proskaüer production from glucose (Buchamann 1990). Other suggested tests are utilization of Xylose, Mannitol, Rhamnose and CAMP test using *Staphylococcus aureus* and *Rhodecoccus equi* and the pathogenecity respectively (Buchanan, 1990).

2.9.3 Typing and Sub-typing of *Listeria monocytogenes and other Listeria* species

Strains of *L. monocytogenes* are subdivided into serotypes based on cellular (O) and flagellar (H) antigens. Eleven serotypes have been designated:

1/2a, 1/2b, 1/2c, 3a, 3b, 4a, 4ab, 4b, 4c, 4d, and 4e. Others are *L. ivanovii* 5, *L. innocua* 6a, 6b, 4ab and not designated *L. seeligeri* 1/2a, 1/2b, 1/2c, 4b, 4c, 4d, 6b not designated *L. welshimeri* 1/2b, 4c, 6a, 6b, and not designated (Weaver, 1989; Lovett, 1990) (Table 4 and Table 5).

Table 4: Serovars of True *Listeria* species

Listeria speies	Serovar
Listeria monocytogenes	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4^{a}, 4ab, 4b, 4c, 4d, 4e, “7”
Listeria innoccua	6a, 6b, 4ab, undesignated
Listeria welshimeri	6a, 6b
Listeria seeligeri	1/2b, 4c, 4d, 6b undesignated
Listeria ivanovii	5

Lovett, 1990

Table 5: Serovars of the genus *Listeria monocytogenes, Listeria grayi* and *Listeria murrayi*

Designation of Seeliger and Donket-Voet	O antigen	H antigen
1/2a	I, II, (III)	AB
1/2b	I, II, (III)	ABC
1/2c	II, (III)	BD
3a	II, (III), IV	AB
3b	(III), IV, (XII, XIII)	ABC
3c	(III), IV, (XII, XIII)	BD
4a	(III), (V), VII, IX	ABC
4ab	(III), V, VI, VII, IX, X	ABC
4b	(III), V, VI	ABC
4c	(III), V, VI	ABC
4d	(III), V. VI. VIII	ABC
4e	(III), V, VI, (VIII), (IX)	ABC
5	(III), (V), VI, (VIII), X	ABC
7	(III), XII, XIII	ABC
6a (4f)	(III), V, (VI), (VII), (IX), XV	ABC
6b (4q)	(III), (V), (VI), (VII), IX, X, XI	
L. grayi	(III), XII, XIV	E
L. Murrayi	(III) XII, XIV	E

( ) = antigens not always present

Lovett, 1990

Most human infections are caused by *L. monocytogenes* strain 1/2a, 1/2b and 4b (Schuchat *et all.,* 1991). Although serotyping is of limited value in epidemiologic investigations, isolates that are of a single serotype have been suggested to be serotyped using isoenzyme or bacteriophage in order to show a more precise comparison of clinical and environmental *Listeria* species (Schuchat *et al.,* 1991; Schuchat *et al.,* 1992).

A phage – typing approach for *Listeria* species has been developed and evaluated for use in epidemiologic studies. Mclauchlin *et al.,* (1986a) tested a set of 28 phages on clinical specimens collected in the United Kingdom (UK) and reported that sixty four percent of *L. monocytogenes* were typeable. This typeability, however, varied in serogroups. They recorded eighty-two percent of serogroup four strains as typeable while only thirty-seven percent of serogroup 1/2a strains were phage-typeable. In previous epidemiological investigation in the United States of America and France other workers had employed phage typing (Audurier *et al.,* 1979; Flemming *et al.,* 1985).

To overcome the problems of phage-typing of some strains, Selander *et al.,* (1986) described multilocus enzyme electrophoresis which distinguishes among *Listeria* isolates of specific *Listeria* genes. By this method all strains of *L. monocytogenes* are typeable. This multilocus enzyme electrophoresis permitted the typing of all strains of *L. monocytogenes* despite the substantial diversity existing among *Listeria* species. This method is very imperative in epidemiologic investigations that may attempt to compare clinical and environmental strains Bibb *et al.,* (1989 and 1990).

The use of restriction fragment length polymorphism (RFLP) analysis of *Listeria* strains has been reported. This method is by using hybridization experiment on the restriction fragments of chromosomal DNA with fibres that recognize relatively stable chromosomal regions (Grimont and Grimont, 1986). This method has some advantages which include: (a) Highly considered ribosomal genes across *phyla*, thus one universal probe can be utilized for the characterization of several organisms. (b) As bacteria usually contain multiple rRNA operons (seven in *E. coli*), adequate number of bands of different molecular sizes are obtained. The ribotyping has further divided the strains into two distinct subgroups – separating types' 1/2a, 1/2c and 3a from serotypes 1/2b and 4b which appeared similar when observed in multilocus enzyme electrophoresis (Grimont and Grimont, 1986).

In a related work, it was reported that ribotyping in conjunction with enzyme typing provided laboratory support that there was a relationship between *L. monocytogenes* isolated from milk and a patient with listeriosis (Vogt *et al.,* 1990). It was discovered that the listeriosis was contracted after the patient had consumed infected milk. Although many methods of subtyping *L. monocytogenes* are good, they are laborious and also could not easily permit rapid screening of large number of *Listeria* isolates Schuchat *et al. (*1991). The question therefore, concerning reproducibility and sensitivity of various methods continues to be searched for in the research and diagnostic Microbiology laboratories.

2.9.4 Molecular Characterization of *Listeria monocytogenes* and Other *Listeria* Isolates by Polymerase Chain Reactions (PCR)

Principle of PCR

The PCR is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers that hybridize to opposite strands of a target sequence. The primers are oriented with their 3 ends pointing towards each other. There was 30 repeated cycles of heat - denaturing of template, annealing of primers, to their complementary sequences and extension of the annealed primers with a deoxyribonucleic acid polymerase (*Thermus aquaticus)* in a Thermocycler machine.This results in the amplification of the segment defined by 5' ends of the PCR primers.

Then the extension product of each primer further serves as template for the other primer. Each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of specific fragments, up to several million-fold in few hours (2-3hours).

The PCR involves three (3) major stages: denaturing, annealing and extension. In denaturing the DNA is melted to convert double stranded DNA to single stranded DNA (2); on annealing, primers are annealed to the target DNA and (3) the DNA is extended by nucleotide addition from the primers by the action of DNA polymerase. The oligonucleotide primers are designed to hybridize to regions of DNA flanking a desired target gene sequence of the organism by the aid of the polymerase (Taq polymerase), in the presence of a free deoxynucleotide triphosphate, resulting in the duplication of the starting target

material. Melting the product DNA duplexes and repeating the process result in an exponential increase in the amount of the target DNA (Figure 3).

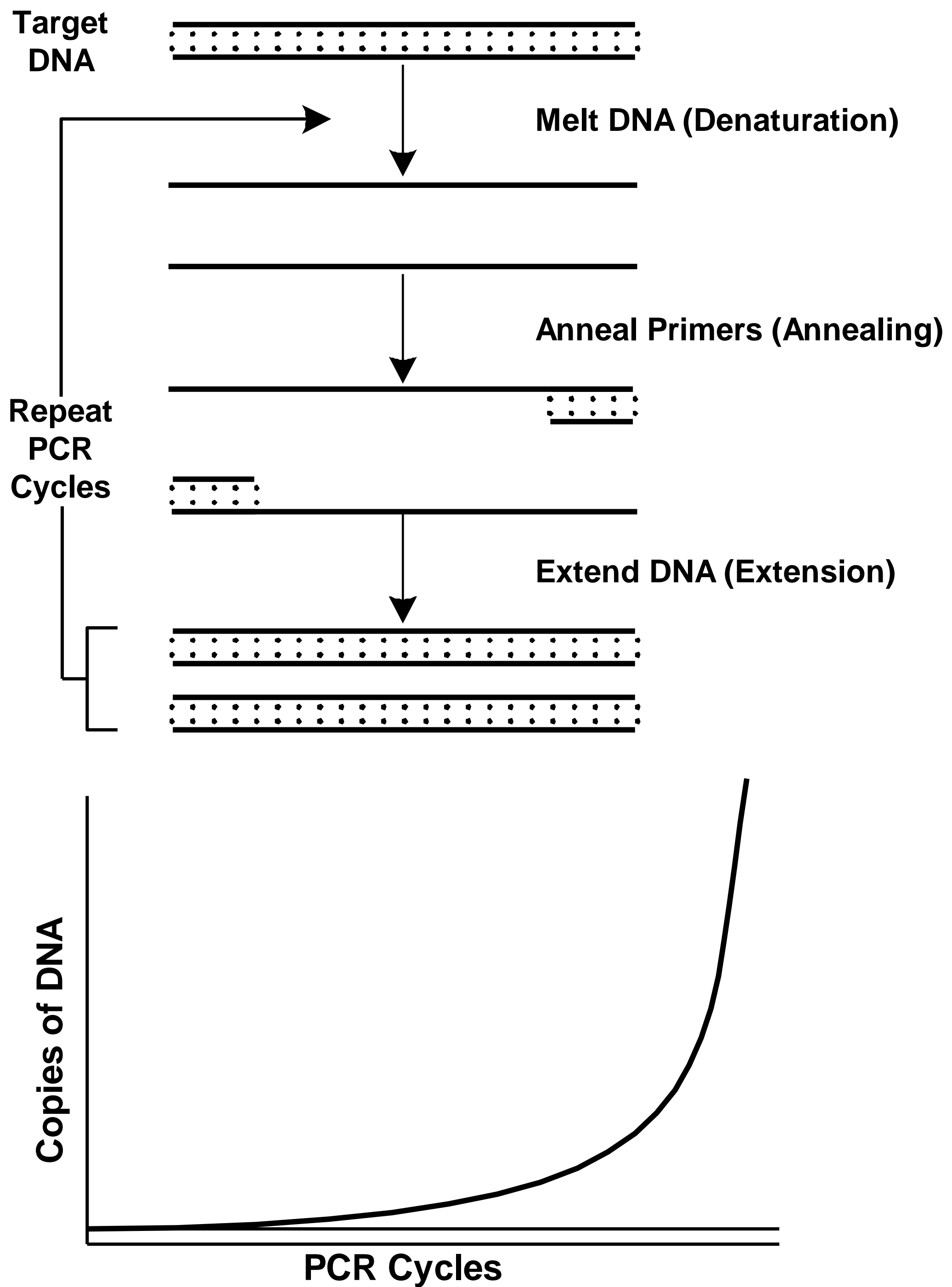

Figure 3: The Stages of PCR and the Resultant Amplification of DNA Copies of the Target Region[1]

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[1] Staffan and Atlas, (1991)

Recent molecular applications in biotechnology are rapidly altering the previous tedious diagnostic procedures used in the field of microbiology, for the isolation and characterization of microorganisms in samples. Pathogenic bacteria are often isolated and identified after much labour, time and utilization of various media. These setbacks can now be circumvented by measuring specific physiochemical changes resulting from the growth of metabolic activity of an organism in a given sample (Swaminathan and Feng, 1994).

The molecular technique using polymerase chain reaction (PCR) methodologies for the detection of bacteria pathogens in clinical, food and environmental samples have been reported (Bej *et al.,* 1990) In PCR, provided that the nucleotide sequence of interest is known, the synthesis of an RNA probe specific for virtually any segment of DNA is possible (Josephson *et al.,* 1991).

This is by using suitable pair of primers for the generation of template, without the need for specific restriction endonuclease sites as required in the recombinant DNA approach. Furthermore, Blais and Phillipe (1993) reported that RNA probe hybridization through PCR is more sensitive than the conventional agarose gel electrophoresis of RNA probe hybridization analysis of PCR products in a wide variety of applications. These include the detection of food pathogens, bacterial and viral diagnosis as well as basic research.

Ever since microbiological analysis was routinely applied to specimens, microbiologists have been trying to simplify or device faster methods for testing or detecting microorganisms from specimens. Most of these efforts, however, consisted of media or procedural modifications that shortened assay time only to

a limited extent. However, the advance in biotechnology has given rise to new diagnostic methods that no longer rely on agar media only (Swaminathan and Feng, 1994). These assays encompass group of systems which include physiochemical tests that measure bacterial metabolites, highly specific DNA – and antibody – based methods, and even fully automated instrumental diagnostic system.

The use of molecular techniques by ribosomal (rRNA) sequences for identification and characterization phylogeny of micro organisms has advanced the study of microbial ecology (Ward *et al*., 1990). Moreso, Polymerase chain reaction (PCR) is a useful technique for the identification and phylogenic characterization of *L. monocytogenes* in environmental food and chemical samples (Bubert *et al*., 1992 and Niederhauser *et al*., 1992 and 1993). The PCR allows specific amplification of the region of DNA to be identified as solely for the strain.

The recombinant DNA techniques have revolutionized molecular microbiology, biology and genetics by permitting the isolation and characterization of specific DNA fragment. Many cloning methods can be complemented and sometimes even circumvented by the use of polymerase chain reactions (PCR). The novel application of PCR technique now permits studies that were not possible before now (Mullis *et al*., 1986; Mullis and Falona, 1987). The method is useful in every type of laboratory interested in molecular biology from forensics to ecology and diagnostics to pure research (Erlich, 1989).

The PCR permits an *in vitro* replication of defined sequences of DNA whereby gene segments can be amplified. By experimentally amplifying a target

sequence, PCR significantly enhances the possibility of detecting rare sequences in heterogeneous mixture of DNA. Thus, PCR is an *in vitro* method for producing large amounts of specific DNA fragments of defined length and sequence from small amounts of a complex template according to (Staffan and Atlas, 1991; Swaminathan and Feng, 1994).

The detection and measurement of DNA sequence variation among bacteria, *L. monocytogenes* and other *Listeria* species is now a routine. This is made possible by the use of techniques such as restriction of enzyme length polymorphism and direct gene sequencing using PCR. These techniques are having major impact on the practice of *Listeria* systematics and molecular epidemiology (Boerlin *et al.*, 1991). In most of the studies on *L. monocytogenes* and other *Listeria* species, molecular markers are being used by many workers in the area of molecular ecology, molecular phylogeny, molecular evolution, taxonomy, behaviour, natural habitat and pathogenicity (Bubert *et al.,* 1992; Goebel *et al.,* 1993; Dramsi *et al.,* 1996).

Studies on the invasion associated protein (*iap*) gene of *L. monocytogenes*, using PCR has been documented (Bubert *et al.,* 1992). The protein has a molecular weight (mw) of 60kiloDaltons (kDt). These workers observed in different *Listeria* species conserved and variable regions within the invasion associated gene which was unique to each particular species. Bubert *et al. (*1992) further reported that the use of PCR for the invasive associated protein (*iap*) gene sequencing along with specific specimen has made it easier and simpler with the identification of *Listeria* species.

In a similar study, Niederhauser *et al.,* (1993) used gene DNA probe PCR in the detection of *L. monocytogenes* from naturally contaminated soft cheese. Considering the sensitivity, specificity and time of analysis, PCR is the most effective means for the detection and identification of the organism in soft and semi-soft foods. From their observation on cheese, Niederhauser *et al.,* (1994) further investigated the use of PCR as a tool in the detection of *L. monocytogenes* in food and clinical samples. During the course of these investigations, they applied a specific oligonucleotide against the listeriolysin O gene of *L. monocytogenes* coupled to para-magnetic beads to isolate *Listeria* DNA from food homogenates and blood.

Denear and Boychuk (1991) had used PCR to detect and specifically identify *L. monocytogenes* from a number of other Gram – positive and Gram – negative organisms. Fifty (50) organisms were routinely detected by the use of PCR. This, however, involves two rounds of thirty- five (35) amplification cycles each without the need of subsequent hybridization with labelled pulses.

They also developed a higher sensitive ligase chain reaction assay based on a single – base pair differences in V9 region of the 16S rRNA gene between *L. monocytogenes* and other *Listeria* species by amplifying the 16S rRNA before the ligase chain reaction. This ligase chain reaction was further tested on 19 different *Listeria* species and strains and proved to be a highly specific diagnostic method for the detection of *Listeria* species in clinical samples of public health significance.

Although molecular phylogeny is profoundly influencing the field of microbiological evolution, taxonomy and detection without culturing them, there are problems associated with the PCR techniques for detecting pathogens in clinical and other samples. These include the difficulty of extraction of organisms or DNAs from such samples. The subsequent purification of the sample is to remove colloidal debris or human substrates which can inhibit PCR (Josephson *et al.,* 1993). Other problems include reagent preparation, primer dimmers, transfer or carryover of DNA and quality control of the PCR.

2.10 IMMUNOLOGICAL ASSAY

In addition to the classical methods using cultural media followed by biochemical and/or serological confirmation, immunoassay methods which offer faster and specific alternatives have been developed. Therefore, developments of methods for immunization of animals and screening them for specific monoclonal antibodies have resulted in monoclonal antibodies that are reactive to live bacteria (Anonymous, 1995).

The use of monoclonal antibodies (Mab) for the detection of *Listeria* species abound. Bhunia and Johnson (1992) reported the use of malls masked with *Listeria* species conserved and variable regions within the invasion associated gene which was unique to each particular species. Bubert *et al.,* (1992) further reported that the use of PCR for the *iap-* gene sequencing along with specific primers has made it easier and simpler in the identification of *Listeria* species.

The accuracy of immunoassay had earlier been demonstrated in the United Kingdom (UK) by food manufacturers (Roberts, 1994). Trial tests on the use of

immunoassay to detect the presence of *L. monocytogenes* on a wide variety of different foods were satisfactory. Some of the foods tested were dairy samples, desiccate and confectionary foods. Others were raw vegetables, dry foods, raw and processed meat and poultry as well as additives (Roberts, 1994). Of the 995 samples tested, 50 were positive results using the immunoassay. Furthermore, of the 50 positive results, 9 were identified as *L. monocytogenes* serotype 4b.

On the intracellular secretions, all species of *Listeria* secrete a major extracellular protein called (P^{60}) due the molecular of 60 kkilodalton. This extracellular protein has been exploited for diagnostic purposes on *Listeria* species. However, an earlier study and comparison of all *Listeria P60* proteins indicated that there were only few regions unique to the pathogenic food-borne species of *L. monocytogenes* (Bubert *et al.,* 1992).

The two unique regions of the *P60* were used for the development of antibodies specific for the detection of *L. monocytogenes* with synthetic peptides by immunization of rabbits. These antibodies were raised against synthetic peptide A (Pep A) and peptide D (pep D) (Bubert *et al.,* 1994). It was observed that both antibodies to pepA and pepD (antisera) detected *L. monocytogenes* in food, clinical and environmental samples (Bubert *et al*., 1994).

In addition to the western blots, antibodies to pepA and pepD reacted specifically with secreted *L. monocytogenes*, *P60* in ELISA analysis. The work revealed the recognition of the native antigen (protein) along side the denatured form. Furthermore, specific detection of secreted P^{60}-protein in the *L. monocytogenes* culture broth supernatant was also possible after an enrichment

of food samples that were contaminated with the organism. Moreso, clinical isolates of *L. monocytogenes* with a typical biochemical characteristic were unequivocally identified using these antibodies. For these reasons, they, therefore, stated that synthetic peptides derived from the variable region of *L. monocytogenes* P^{60} are useful for the production of highly reactive antibodies (antisera) that can be applied in the diagnostic approaches of *L. monocytogenes* and other *Listeria* species in food, environment and medical probes. The use of *P60* as the main target molecule for immunological detection of *L. monocytogenes* has been reported (Bubert *et al.,* 1994).

The advantages of P^{60} in biosciences are enormous due to its Sensitive, specificity, reliability, availability and safety (Bubert *et al.,* 1992; Rhuland *et al.,* 1993; Wuenscher *et al*., 1993; Bubert *et al.,* 1994*).*

2.11 EPIDEMIOLOGY OF *LISTERIA* rORGANISMS

2.11.1 *Listeria monocytogenes* in the Environment: Soil, Water and Vegetation

Listeria monocytogenes and other *Listeria* species have been isolated from various environments such as human and animal wastes. *L. monocytogenes* has been isolated from soil, water, farmland and decaying vegetation (Welshimer, 1968; Weis and Seeliger, 1975). Weis and Seeliger (1975) isolated *L. monocytogenes* from 21% of 779 plant and soil samples. They reported serotypes 1/2b and 4b to be most frequent.

The ability of the *L. monocytogenes* bacteria to survive in environmental sites like sewage, river water and sewage sludge has been demonstrated quantitatively (Watkins and Sleath, 1981). They workers reported that agricultural lands

remained unchanged for at least eight weeks harbouring large quantities of the organism. The potential implications of faecal material as agricultural fertilizer became evident when this practice was linked to a large outbreak of human listeriosis in Nova Scotia (Schlech *et al.,* 1983).

2.11.2 Transmission of *Listeria monocytogenes*

The transmission of *L. monocytogenes* to man and animals has been linked to food intake since the early fifties in Central Germany, other European countries and United States of America (Gray and Killinger, 1966; Seeliger, 1988; Adak *et al*., 2005). For the onset of listeriosis, the organism first penetrates through the intestine. This is followed by multiplication of the organism in various types of the host cells intracellularly. However, the epidemiology of human listeriosis had remained obscure until in the recent past. This was especially due to sporadic cases of listeriosis observed among veterinarians and farmers, traced to the animals they handled (Seeliger, 1988).

Although, there may be clinical and microbiologic aspects of *L. monocytogenes* and other *Listeria* species known, Gellin and Broome, (1989) reported that the knowledge of the epidemiology of human listeriosis is far from being complete. Gellin and Broome, (1989) observed that many factors, including the incidence and mode of transmission, have remained relatively obscure. This, they argued, might be as a result of listeriosis being a rare disease or under reporting of the disease. Therefore, as a result of this, an incidence of the disease in a community may go undetected except by chance.

2.11.3 *Listeria monocytogenes* in Animals

Since Murray *et al.,* (1926) described the disease–listeriosis in rabbits, caused by *L. monocytogenes* it has generally been thought as a veterinary disease. In mammals, *L. monocytogenes* causes abortions and "circling disease" (meningoencephalitis), and epizootics of listeriosis have been observed in herds of cattle and sheep long before outbreaks of human listeriosis were recognized (Gill, 1925).

Listeria monocytogenes has been isolated from cattle, sheep, pigs, chickens, ducks, and a variety of other species (Gray and Killinger, 1966). In addition, *L. monocytogenes* causes diseases in some 60 or more species of wild and domesticated, warm and cold blooded animals. The organism has been considered as zoophilic, and the various syndromes were ascribed to zoonosis (Gray and Killinger, 1966; Blenden *et al.*, 1987).

By systematic culture of stool from cow with *Listeria*-related abortions, from healthy cows in herds with listeriosis and from cows in unaffected herds, Bojsen–Moller (1972), reported that there were differences in the carriage rate of *L. monocytogenes*. The highest rates of carriage were found in stool cultures of animals that had aborted due to listeriosis 53 out of 219 (24%) samples. On the other hand, healthy cows from affected and unaffected herds had lower rates of *Listeria* carriage than affected herds which had 6.6 %(41 out of 622) and unaffected herds 1.7% (Bojsen-Moller, 1972).

The relationship between the feeding of domesticated ruminants on poor – quality silage and the onset of listeriosis has been reported. Palson, (1963)

reported that silage of poor quality may harbour enough *L. monocytogenes* to cause infection in dairy cattle. This he explained causes more prevalence of listeriosis especially during stabling period when the animals are fed with silage. Blenden and Szatalowicz (1967) also reported that in addition to weather extremes, deprivation of food or water, parasites, metabolic stress, transportation and use of steroids or endemic related changes may alter the epidemiology of animal listeriosis. Nevertheless, clinical illness and asymptomatic carriage of *L. monocytogenes* are well documented in animals.

2.11.4 *Listeria monocytogenes* in Humans

Before, listeriosis was known to be zoonotic disease, human listeriosis was thought to be due to illness occurring in animals, and the animal host was considered as the primary reservoir for *L. monocytogenes* (Bojsen-Moller, 1972). Contrary to the earlier reports, epidemic and sporadic cases listeriosis has confirmed the disease as zoonotic (Schlech et al., 1983; Cain and McCann, 1986; Schuchat *et al.*, 1991, 1992; Chukwu *et al.,* 1997; Dyer and Stoltenow, 2002).

In some reported cases of human listeriosis, there is seldom history of direct contact with animals. Owing to this, Gray and Killinger (1966) argued that most human listeriosis occurred in urban residents, while only rare cases occurred among residents of rural areas where domestic animals were widely affected by listeriosis. However, the idea that human listeriosis could be the result of indirect contact with infected animals focused attention on the possibility of transmission of *L. monocytogenes* by food. Thus, Gray and Killinger (1966) suggested a possible listeric infection cycle of Man and Animals as: infected animals/humans

→ animal/human faeces → soil contamination → soil or faecal contamination of crops and vegetables → oral infection of animals/humans.

In livestock and man, Gronstol, (1986), Figure 4; Blenden *et al*., (1987), Figure 5; Audurier and Martin (1989), Figure 6; Topley and Wilson (1990) Figure 7; reported possible infection cycle as: infected animals and humans → shedding of *L. monocytogenes* with urine and faeces → organisms persisting in soil, manure, dust or filth.

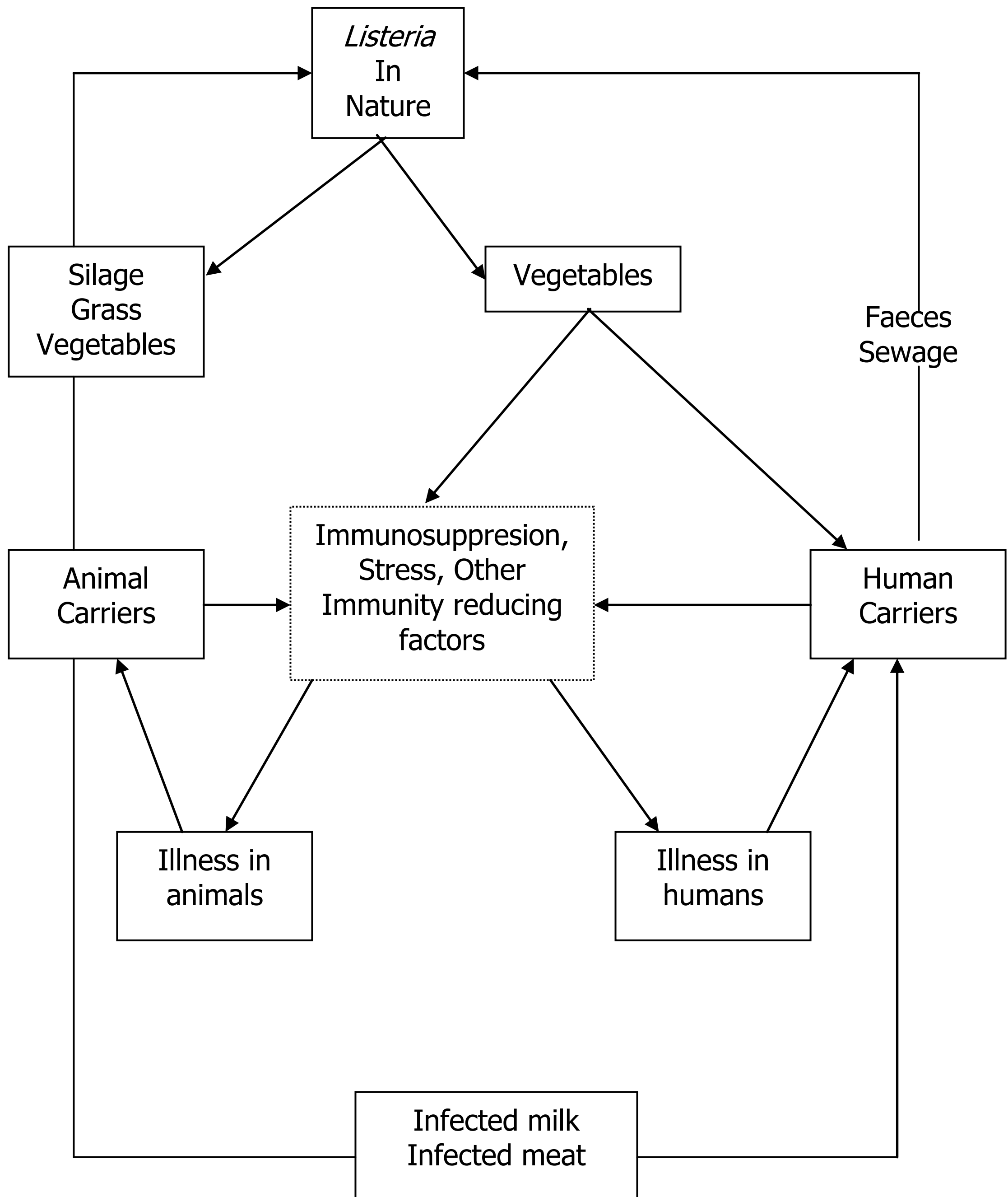


Figure 4: Routes of contamination and infection with *L. monocytogenes* (Gronstol, 1986)

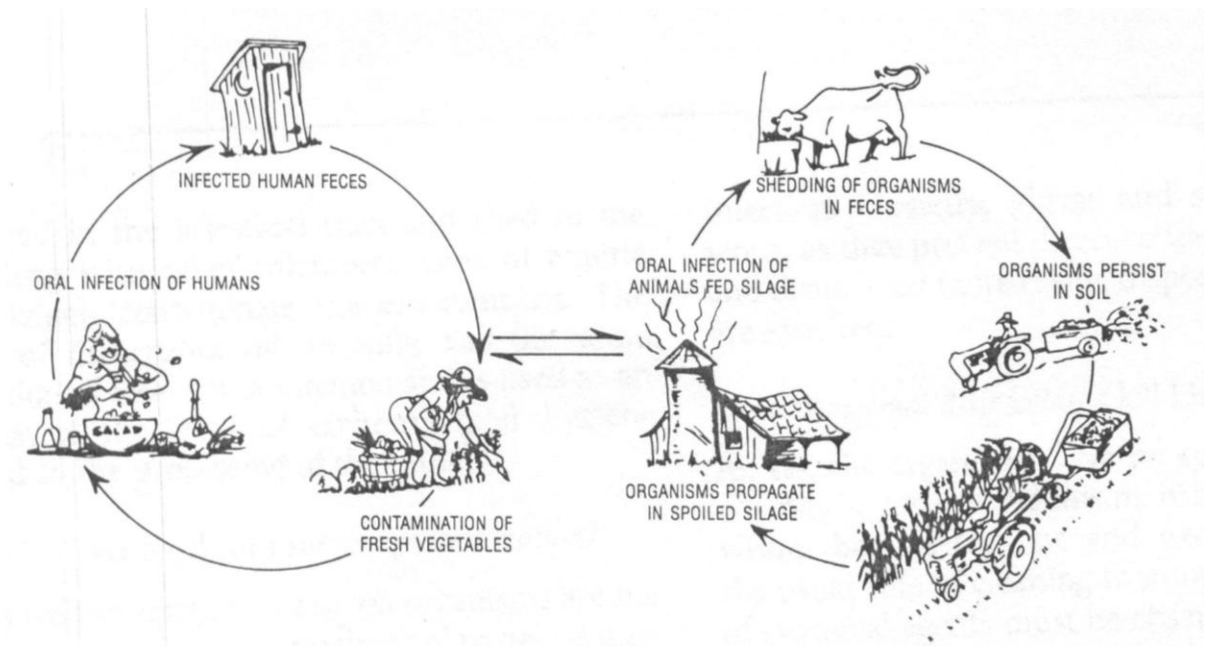


Figure 5: Possible *Listeria* Infection Cycles in Man and Domestic Animals (Blenden *et al.*, 1987)

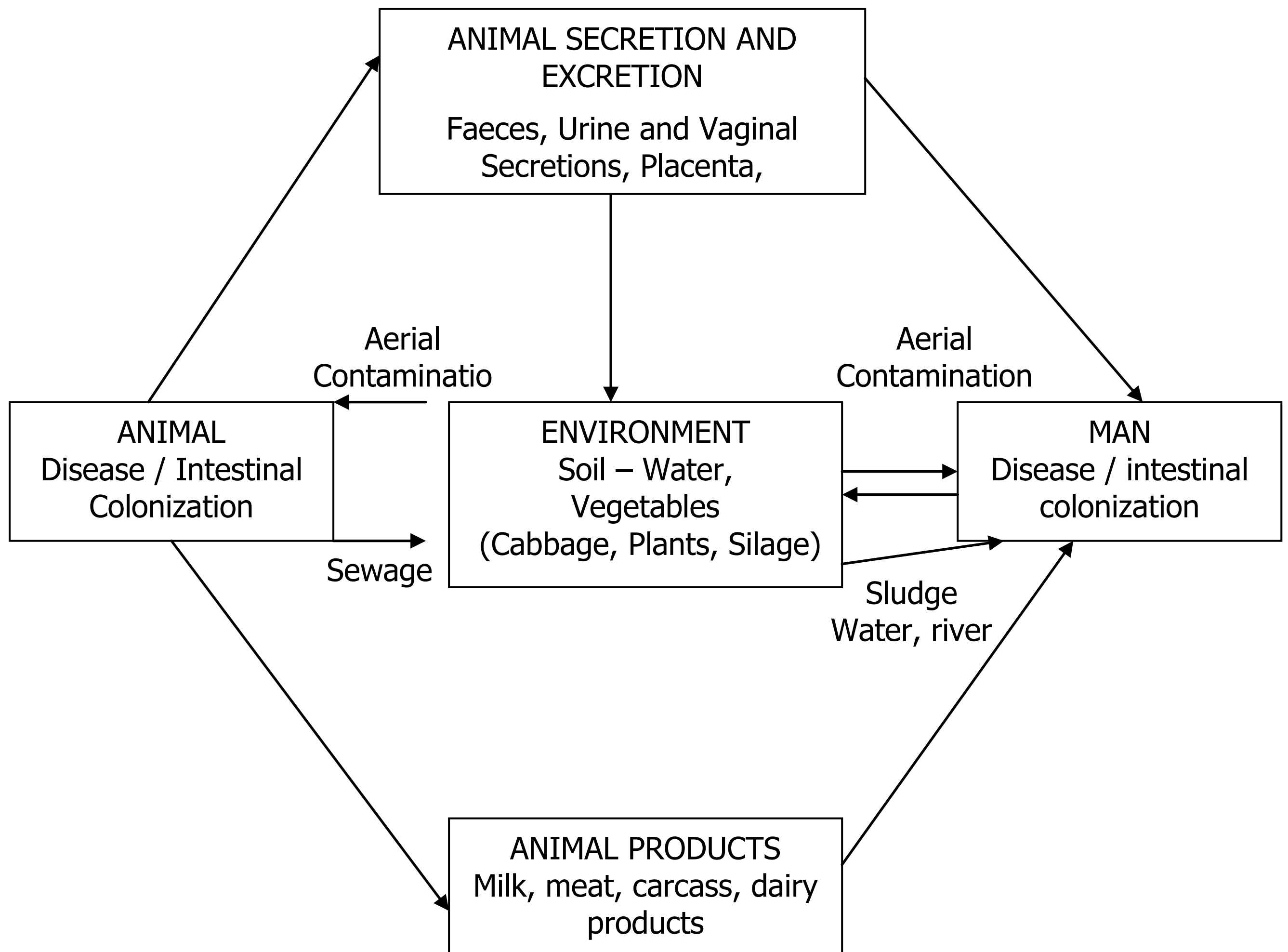


Figure 6: Ways in which *L. monocytogenes* is disseminated in the environment, animals, foods and humans

(Audurier and Martin, 1989)

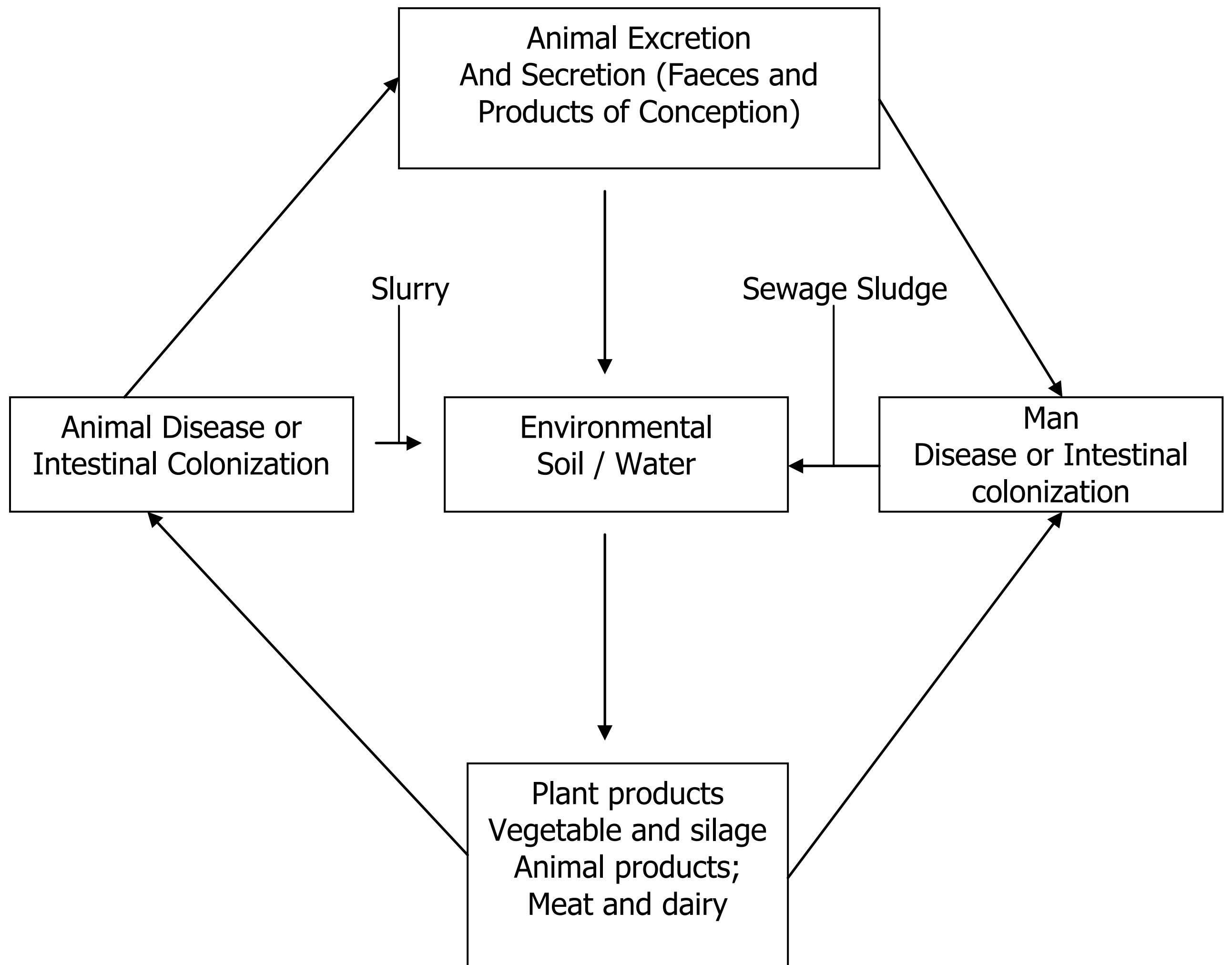


Figure 7: Different Ecological Interactions of *Listeria* Species and Biological Interactions in Listeriosis

(Topley and Wilson, 1990)

Propagation under favourable environmental conditions such as neutral or alkaline silage leads to oral infection of the animal through silage. However, *L. monocytogenes* may be isolated frequently from the environment or from food without a direct relationship to human illness. In the past, initial typing systems were not sufficiently precise to suggest that environmental strains were related to clinical isolates.

However, the investigation of large outbreaks of human listeriosis finally provided epidemiologic and laboratory support to confirm the suspicion that listeriosis was a food-borne disease (Schlech *et al.,* 1983; Flemming *et al.,* 1985; Bille 1990; Vogt *et al*., 1990; Farber and Peterkin, 1991).

Since the report of Murray *et al.,* (1926), it has been suggested that the alimentary tract might be the portal of entry for the organism causing listeriosis. Several investigations have found *L. monocytogenes* in faecal specimens from healthy individuals (Kampelmacher and Van-Noorle Jansen, 1969; Bojsen–Moller, 1972). Kampelmacher and Van Noorle Jansen (1969) reported 11.9% faecal *Listeria* carriage and slaughterhouse workers had a rate of 13.3% and Bojsen–Moller (1972) reported faecal carriage of the organism in a number of population groups. He recorded *Listeria* in faecal cultures from healthy slaughterhouse workers (4.8% out of 1,147 workers sampled). Thus, there is a consistent fact with the suggestion that the gastrointestinal tract is the human reservoir of *L. monocytogenes*.

Other studies from various pregnant and non- pregnant individual groups have identified wide ranging estimates for stool carriage of *L. monocytogenes*

among healthy adults. In a similar investigation, Vogt *et al.,* (1990) found *L. monocytogenes* in 1.75% of stool samples from 400 patients hospitalized with non listeric conditions. However, differences in the prevalence carriage have been reported when serial specimens from individuals were cultured (Schuchat *et al.,* 1991a; Dyer and Stoltenow, 2002).

2.11.5 Epidemiology of *Listeria monocytogenes* Infections

In 1981, a large outbreak of Listeriosis occurred in Nova Scotia, Canada. This outbreak provided the first documented evidence that *Listeria* infection / transmission is often through food (Schlech *et al.,* 1983). The outbreak was recognized when perinatal listeriosis occurred in (1.3%) of 97 births at the maternity hospital with a fatality rate as high as (27%) among live born infants and 19 intrauterine deaths.

However, none of the non-pregnant adults infected had any evidence of underlying immunosuppressive conditions. Among the adults infected, two deaths were recorded. In this outbreak episode, a case-study implicated locally made coleslaw as the possible vehicle of transmission. During the investigation, *L. monocytogenes* serotype 4b of the epidemic (outbreak) strain was the same as the strain isolated from the cole-slaw (Schlech *et al.*, 1983).

On further investigation, it was reported that ovine listeriosis had been diagnosed on the farm of which the manure was in turn used to fertilize the cabbage farm. This cabbage implicated served as a raw material for the coleslaw product. These findings revealed that the cabbage must have been contaminated in the field during cropping with the manure. Thus, cold storage of the cabbage

for long time enhanced the multiplication of *L. monocytogenes* before consumption in coleslaw product (Schlech *et al*., 1983).

In a related development in 1981, 11 cases of listeriosis were also reported in Carlisle, East Columbia and the United Kingdom. In this episode, seven adults and four neonates were affected. The serotype implicated was *L. monocytogenes* serotype 1/2a, which is an unusual serotype for an outbreak of listeriosis (Mclaunchlin *et al.,* 1986b). Also during the investigation of the episode, no mode of *Listeria* transmission was recorded. Also in Germany, within the first 9 months in 1983, 25 cases of human listeriosis were reported in Saxony. The implicated *L. monocytogenes* was serotype 4b (Nicolair and Scholter, 1985).

Similarly, another listeriosis outbreak implicated raw vegetables in Boston (Ho *et al.,* 1986). During the outbreak, studies revealed that the patients were likely to have eaten tuna-fish, chicken – salad and cheese often served with a raw – vegetable garnish. On culture of the patients' samples, *L. monocytogenes* serotype 4b was isolated. Also, when the vegetables were screened for the organism the same serotype 4b was isolated. It was then speculated that the raw vegetable might have been responsible for the outbreak. However, in contrast, no single common food exposure was implicated (Ho *et al.,* 1986; Sizmur and Walker, 1988).

Fleming *et al.,* (1985) reported an outbreak of listeriosis which occurred in Massachusetts in 1983. This outbreak involved non pregnant adults who had immunosuppressive condition. At the period of the outbreak, there were

simultaneous diagnostic cases of listeriosis in foetuses and neonates with fatality rate of 29% in both adults and neonatal groups.

An investigation implicated pasteurized milk as the possible cause of the epidemic but milk from the pasteurization plant yielded no *L. monocytogenes* serotype 4b, like the epidemic strain. However, raw milk from the farms that supplied milk to the plant yielded *L. monocytogenes* (but not the epidemic strain type). Thus, Fleming *et al.,* (1985) concluded that post pasteurization contamination was one most likely explanation for the outbreak.

One of the largest listeriosis outbreaks in the USA was reported by Linnan *et al.,* (1988). In this outbreak, majority of the cases were in pregnant Hispanic mothers and their neonates. Out of 93, 81(87%) cases were pregnant-associated resulting to 29 foetal or neonatal deaths. A Mexican–style soft cheese was implicated as the vehicle of the transmission. The epidemic strain was isolated from a number of unopened packages of the suspected cheese. The contamination of the cheese was said to have resulted from unpasteurized milk incorporated into the finished product.

The outbreak revealed that sporadic listeriosis might be the outcome of a single ethnic group who shared common medical facilities or contaminated food sources and that the clustering of cases might not be recognized if the transmission vehicle was widely distributed or if the patients present themselves to different medical facilities (Linnan *et al.,* 1988).

Also, an unusual outbreak of listeriosis was reported by Schwartz *et al.,* (1989) in Philadelphia metropolis area of USA, involving mostly non- pregnant adults with immunosuppressive conditions and the elderly. A patient–case study showed that the patients had, had an ill family member using antidiarrhoeal medication before their listeriosis episode. Also, dietary investigation revealed that ice-cream and salami purchased from one grocery store chain eaten by the patients were responsible.

However, cultures of the food products that were consumed by the patients were negative for *L. monocytogenes* except Brie cheese eaten by one of the patients with no predominant strain of *L. monocytogenes* after investigation, Schwartz *et al.,* (1989). It was adjudged that a common source of contamination of the food products and a combination of *L. monocytogenes* carriage and a co-infecting organism could have precipitated the dissemination of the Listeric infection in this outbreak.

Besides the USA continent, the Europe continent has also experienced several outbreaks and clusters of cases of listeriosis since 1975. From 1975 to 1976 an epidemic was recorded in France with a total of 167 reported cases in two hospitals (Carbonnelle *et al*., 1978). During this epidemic, 76% cases were maternofoetal with a lethality rate of 39% and the predominant strain was *L. monocytogenes* serotype 4b. On investigation, no common source was implicated as the possible source of the infections.

Besides the United Kingdom, France and Germany, the incidence of human listeriosis has been on the increase in Europe. The outbreaks in Denmark,

Australia and Switzerland have been associated with food contamination. In the western part of Switzerland, Piffaretti *et al.,* (1989) reported that 122 cases, resulting in 34 deaths occurred between 1983 and 1987. During this outbreak, over 80% of the human isolate strains, serotype 4b were similar by sero – and phage typing. On investigation of the epidemiology, the same strains were repeatedly cultured from the surface of a brand of locally made cheese.

On investigation of the Switzerland outbreak strains, Piffaretti *et al.,* (1989) reported that the Switzerland outbreak strain had the same electrophoretic enzyme type with that of Mexican style cheese earlier isolated and reported by Schlech *et al.,* (1983) in the United States of America. The comparative analysis of the isolates during the Switzerland epidemic and that of USA in 1983, revealed that 38 out of 40 (95%) of the strains during the outbreak belonged to serotype 4b as against 60% of the strains isolated before 1983–1987 outbreak (Piffaretti *et al.,* 1989).

2.12 NOSOCOMIAL LISTERIOSIS INFECTION

In addition to the large outbreaks of listeriosis due to contaminated food, nosocomial clusters of listeriosis have been reported that were not of food contamination. Simons *et al.,* (1986) on epidemiologic and laboratory investigations, reported a case of an infant delivered with early onset of listeriosis, which subsequently infected other infants in the area. On further investigation of most of the cases, revealed a person–to–person transmission due to poor hygiene. They concluded that a break in barrier nursing technique was possible

sources of the infection as the source of the infection revealed that the disease originated from the mother of the suspected infant (Simons *et al.*, 1986).

In a related outbreak, a larger nosocomial clusters of listeriosis occurred in Costa Rica (Schuchat *et al.,* 1991b). During this outbreak, over 3% of infants born developed listeriosis. The epidemiologic and laboratory investigations proved that the outbreak was as a result of contaminated mineral oil commonly used to bath newborns. Apart from the newborns bathed with this contaminated oil, infants born by caesarean sections also developed the disease. The authors speculated that the infection might have occurred when infants aspirated the contaminated oil applied on their faces or when the oil came in contact with mucous membrane surfaces.

The respiratory portal of entry was evident by respiratory presenting symptoms in many infants. On post mortem, lipid–laden macrophages consistent with oil were present in the lung tissues of the infants who died as a result of the infection (Schuchat, *et al.,* 1991b). On further investigation, the general anaesthesia used in the cases of Caesarean deliveries was found to have increased the infants' risks of aspirating the oil which the babies were exposed to, shortly after birth. This Nosocomial outbreak showed the ability of *L. monocytogenes* to persist in the environment once introduced, confirming the important role of multiuse of materials in the transmission of infectious diseases in delivery rooms.

2.13 SPORADIC LISTERIOSIS IN HUMANS AND ANIMALS

In contrast to the epidemics and seldom nosocomial outbreaks of listeriosis in human and animals, the disease occurs sporadically. Seasonal pattern of occurrence of listeriosis has been reported especially during winter and spring in the U.S.A and Europe (Blenden and Szatalowicz 1967; Blenden *et al.,* 1987).

Infection among animals is often associated with improperly prepared silage used in winter (Fenlon, 1986; Blenden *et al.,* 1987) while human listeriosis has been reported to be mostly common during summer months in Europe and the U.S.A. (Blenden *et al.,* 1987; Gellin and Broome 1989). However, Gellin and Broome, (1989) reported a consistent seasonality occurrence of the disease all year round in the United States of America.

In addition to sporadic listeriosis, dietary risk factors for sporadic, Nosocomial or epidemic have been reported. Schwartz *et al.,* (1988) conducted active surveillance that compared diet and persons with listeriosis. They observed that the age, underlying illness and pregnancy were predisposing factors for those who have eaten undercooked hot dogs which were contaminated with *L. monocytogenes*. The World Health Organization, (1988) report proves the risk exists as *Listeria monocytogenes* was isolated from 20 to 60% of livestock, 15 to 80% of poultry samples and 30% of ready to eat meat products.

Stool or faecal carriage studies by Kampelmacher and Von Noorle (1972) supported the concept that many people may be exposed to *L. monocytogenes* and remain asymptomatic. Thus, by the time the infection is evident, the suspected or implicated food may not be available for microbiologic analysis.

However, subsequent microbiologic investigation of patients and food in cases of sporadic listeriosis has provided support for epidemic studies on listeriosis (Schuchat *et al*., 1992; Pinner *et al*., 1992; Bailey *et al.,* 2003).

Barnes *et al.,* (1989); Wenger *et al.,* (1990) had reported listeriosis associated with the consumption of microwaved turkey franks. Microbiologic infection of the production factory resulted in the detection of the same strain of *L. monocytogenes* on the environmental surfaces and in the final container packages ready for sale to the general public (Wenger *et al.,* 1990).

Another case of listeriosis was linked to the consumption of raw milk (Vogt *et al.,* 1990). In this case the patient was in the habit of drinking raw milk. *Listeria monocytogenes* serotype 1/2a was isolated from the raw milk.The isolate was the same as the one from the 76 year old patient. Characterization of the isolates from the milk and patient by enzyme and ribosoma (rRNA) typing, suggested that the patient must have been infected through the consumption of the contaminated milk (Vogt *et al.,* 1990).

While epidemic and sporadic listeriosis in humans and animals are as a result of food contamination and other materials have been documented in advanced or industrialized countries of Europe, America and others, such reports in Africa are scarce or non- existent (WHO, 1988). The question is whether, this is due to geographical location, different dietary habits or it represents lack of documented medical and veterinary reports.

In West Africa, *L. monocytogenes* has been isolated in animals in Togo and Senegal (Hohne *et al.,* 1975; Baylet and Diop, 1975). However, in Nigeria, Onyemelukwe, *et al*., (1983) Enurah, *et al.,* (1988), Oni, *et al.,* (1989) and Chukwu (1995) have reported *L. monocytogenes* in human, livestock, poultry and food but not in the environment nor has any food been incriminated as sources associated with the infections.

Onyemelukwe *et al.,* (1983) reported the isolation of *L. monocytogenes* from patients who had meningitis, meningoencephalitis, spontaneous peritonitis, septicaemia, arthritis and pelvic infections. The epidemiologic and laboratory findings revealed that the patients may have contracted the disease through animals or animal products as most of the patients were cattle rearers. Also, Oni *et al.,* (1989) isolated *L. monocytogenes* serotype 4b from cattle and milk in Zaria.

In Plateau State, Enurah *et al.,* (1988) reported an outbreak and sporadic listeriosis in National Veterinary Research Institute, Vom farm located in Jos South Local Government Area. There was high mortality rate during the outbreak of the disease. In another laboratory based surveillance on meat and dairy products – 'suya' (Nigerian roasted beef or muttom), Meat loaf, salami, beef frank, hard cheese and pasteurized milk on sale in Jos metropolis of Plateau State, Chukwu (1995), reported a high percent of *L. monocytogenes* from these products sampled. On further characterization by polymerase chain reaction, most of the isolates were serotypes 4a, 4b, 1/2a. Of interest is that these serotypes are the types that have been incriminated in listeriosis outbreaks in Europe and the U.S.A.

Also, *L. monocytogenes*, serotype 4b has been isolated from a dairy animal attendant in an integrated dairy farm earlier diagnosed of listeriosis in Plateau state (Chukwu *et al.,* 1997). This case of listeriosis was confirmed as the extension of the animal listeriosis in the farm. This also revealed the hazards of animal attendants in farms that may have undiagnosed listeriosis.

2.14 PATHOGENESIS OF *LISTERIA* INFECTION

The natural route of entry for a *L. monocytogenes* in man and animals is through the gut (Racz *et al.,* 1972). The cellular invasion and unrestricted replication of the organism in Peyers patches will in most cases ultimately lead to disseminated infection (Njoku-Obi *et al.*, 1963; MacDonald and Carter, 1980). This invasion of the epithelial cells by *L. monocytogenes* constitutes an important early step of infection. More so, virulent *L. monocytogenes* are able to leave phagolyzosome mainly by listeriolysin (LLO) secretion, as well as the introduction of assembly of cytoskeletal elements to propel themselves (*Listeria* organisms) into next cells of hepatocytes, without leaving their intracellular habitat (Portnoy *et al.,* 1988; Tilney and Portnoy, 1989; Mielke *et al.*, 1993).

Host-parasite relationship is manifold. Pathogenic microorganisms find appropriate niche where they can survive within the host. Some strictly or facultatively reside and multiply within the cells of their host, for their parasitic life and others live within as well as outside the host cells (Hof, 1991a).

Intracellular bacteria that cause infectious diseases in humans and animals possess the ability to invade non-professional phagocytic mammalian cells. The bacteria such as *Listeria monocytogenes* and other *Listeria* species, *Salmonella*

species*, Legionella* species and *Mycobacterium* species can grow even inside phagocytic cells after being phagocytosed (Goebel *et al.,* 1991). These microorganisms encounter many obstacles on their way to the intracellular compartments of their host. However, each of these pathogens has developed different mechanisms to circumvent the non-specific and specific defence mechanism pathways of the host(s) for survival and multiplication (Hof, 1991b).

Phagocytosis by the neutrophils and macrophages represent the cornerstone in resistance. These cells destroy the phagocytosed particles (bacteria) by an oxidative burst producing hydrogen peroxide (H_2O_2) and oxygen (O_2) radicals. This is achieved by decreasing the internal pH value and by vacuole and activation of their contents resulting in strong antimicrobial activities (Hof, 1991b).

Nevertheless, some organisms like *Listeria* are not killed and thus, survive due to their ability to neutralize the adverse effect of H_2O_2 and O_2. Thus phagocytosis of bacteria (*Listeria*) activates the enzyme NAD (P) H oxidase which is located in the plasma membrane of mononuclear phagocyte. This enzyme further catalyses the production of superoxide anion (O_2^-) from O_2. The superoxide dismutases on acting on O2-, more toxic residual anions (OH^-) are produced. This toxic radicals formed are further catalysed by an enzyme hydrogen peroxidase to Oxygen and water ($H_2O + O\uparrow$).Thus, the *Listeria* organisms continue to multiply intracellularly (Hof, 1991b).

Phagocytosis by neutrophils and macrophages represents a cornerstone of host-resistant or susceptible of micro organisms (Hof, 1991b). These neutrophils

and macrophages destroy the internalized bacterial particles by an oxidative burst producing hydrogen peroxide (H_2O_2) and oxygen radicals (O^-) under aerobic condition. This decreases the initial pH value and fusion of the lyzosomes with the phagocytic vacuole, and activation of their contents which exert strong antimicrobial activities (Hof, 1991b).

The advancement and application of modern genetic techniques, especially transposon mutagenesis and gene cloning, combined with suitable *in vitro* and *in vivo* model systems, have provided an insight into the multifactorial nature of *L. monocytogenes* pathogenesis and pathogenicity (Gailard *et al*., 1987; Portnoy, *et al.,* 1992a). By using tissue culture models of infection, the cell biology of *L. monocytogenes* has been characterized at the morphological level (Gailard *et al*., 1987; Tilney and Portnoy, 1989 and Dabiri *et al*., 1990).

Subsequent to internalization, the bacteria escape from host vacuole and enter the cytoplasm, where rapid growth ensues. Shortly thereafter, the bacteria appear to mediate nucleation of host actin filaments which rearrange to form a tail consisting of short filaments and actin binding proteins.

With the aid of video microscopy, the bacteria have been observed moving through the cytoplasm at rates of 1.5µm/s (Dabiri *et al*., 1990). Dabiri *et al*., (1990) then hypothesized that actin polymerization is directly required for the movement, as cytochalasin D causes immediate cessation of movement. Some of the bacteria, however, move to the surface of the cell and are extruded from the cell in pseudopod-like structures. The pseudopods are apparently recognized by the neighbouring cell and phagocytosed, where upon the bacteria have to escape

from the resulting double-membrane vacuole in order to enter the cytoplasm again. This model provides a cell biological explanation for the classic observation that antibody plays little or no role in the immunity of *L. monocytogenes*. The steps of the invasion of host cells and intracellular spread of *L. monocytogenes* are shown in Figure 8.

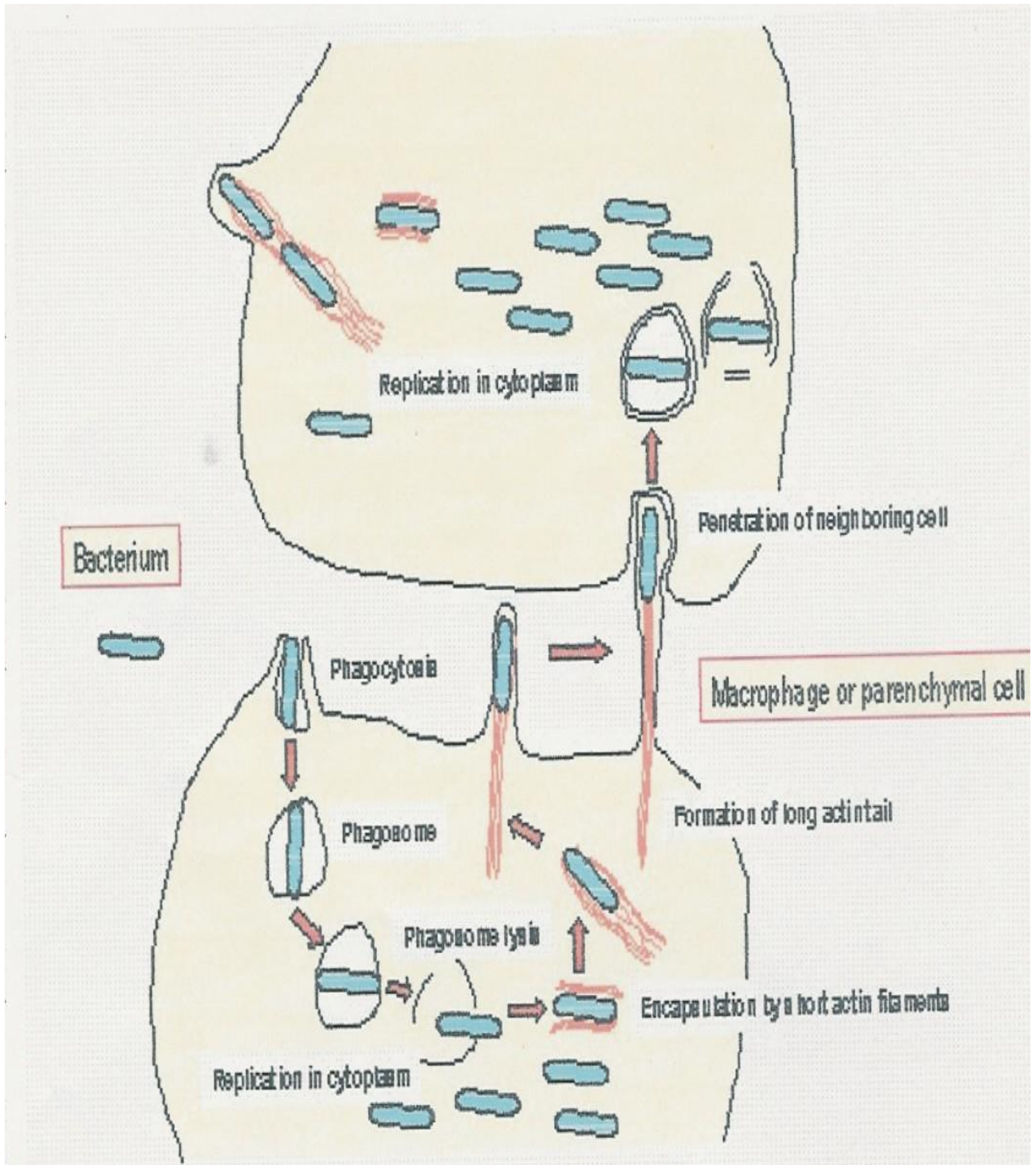


Figure 8: Steps in the invasion of cells and intracellular spread by *L. monocytogenes*

The bacterium apparently invades via the intestinal mucosa. It is thought to attach to intestinal cells by means of D-galactose residues on the bacterial surface which adhere to D-galactose receptors on susceptible intestinal cells. The bacterium is taken up (including by non phagocytic cells) by induced hagocytosis, which is thought to be mediated by a membrane associated protein called internalin. Once ingested, the bacterium produces listeriolysin O (LLO) to escape from the phagosome. The bacterium then multiplies rapidly in the cytoplasm and moves through the cytoplasm to invade adjacent cells by polymerizing actin to form long tails (Dabiri *et al.,* 1990).

Host cell infection begins with the internalization of the organism either by phagocytosis in the case of macrophages, or by induced phagocytosis in the case of nonphagocytotic cells (Tilney and Tilney, 1993). Shortly after *L. monocytogenes* makes contact with macrophages, it is immediately phagocytosed.

Inside the phagosome, Camilli *et al.,* (1991) reported that the organism secretes haemolysin and phospholipase C enzymes. These enzymes will then breakdown the phagosomal membrane and the organism enters the macrophage cytoplasm, where it divides using the host cells nutrients. At the same time, the organism assembles on its surface numerous short chain actin filaments which form a polar tail of about 5μm long as shown in Figure 8. Therefore, in about 2.5 hours of *Listeria* infection, the organism begins to migrate around the cytoplasm of the cell at speeds proportional to the length of their actin tail (Theriot *et al.,* 1992).

As the organism makes contact with the plasma membrane of the macrophage, a protuberance is generated. Then the membrane is applied tightly around the *Listeria* and its tail, like a finger in a rubber glove. When this long pseudopod protuberance makes contact with a neighbouring macrophage, the next macrophage phagocytoses the pseudopod of the first macrophage (Tilney and Tilney, 1993; Kocks, 1994). Thus, the next macrophage is a phagosome containing the plasma membrane that covered the pseudopod of the first membrane. This doubly encapsulated *Listeria* escapes into the cytoplasm by dissolving both membranes, again mediated by phospholipases and haemolysin. This cycle is repeated as the organism migrates as had been shown in Figure 8.

Once *Listeria* has entered the cytoplasm of a macrophage, it can spread from cell to cell without ever again leaving the cytoplasm or entering an extracellular compartment of the host cells. The organism avoids being detected by circulating antibodies (Tilney and Tilney, 1993; Torensma *et al.,* 1993; Kocks, 1994).

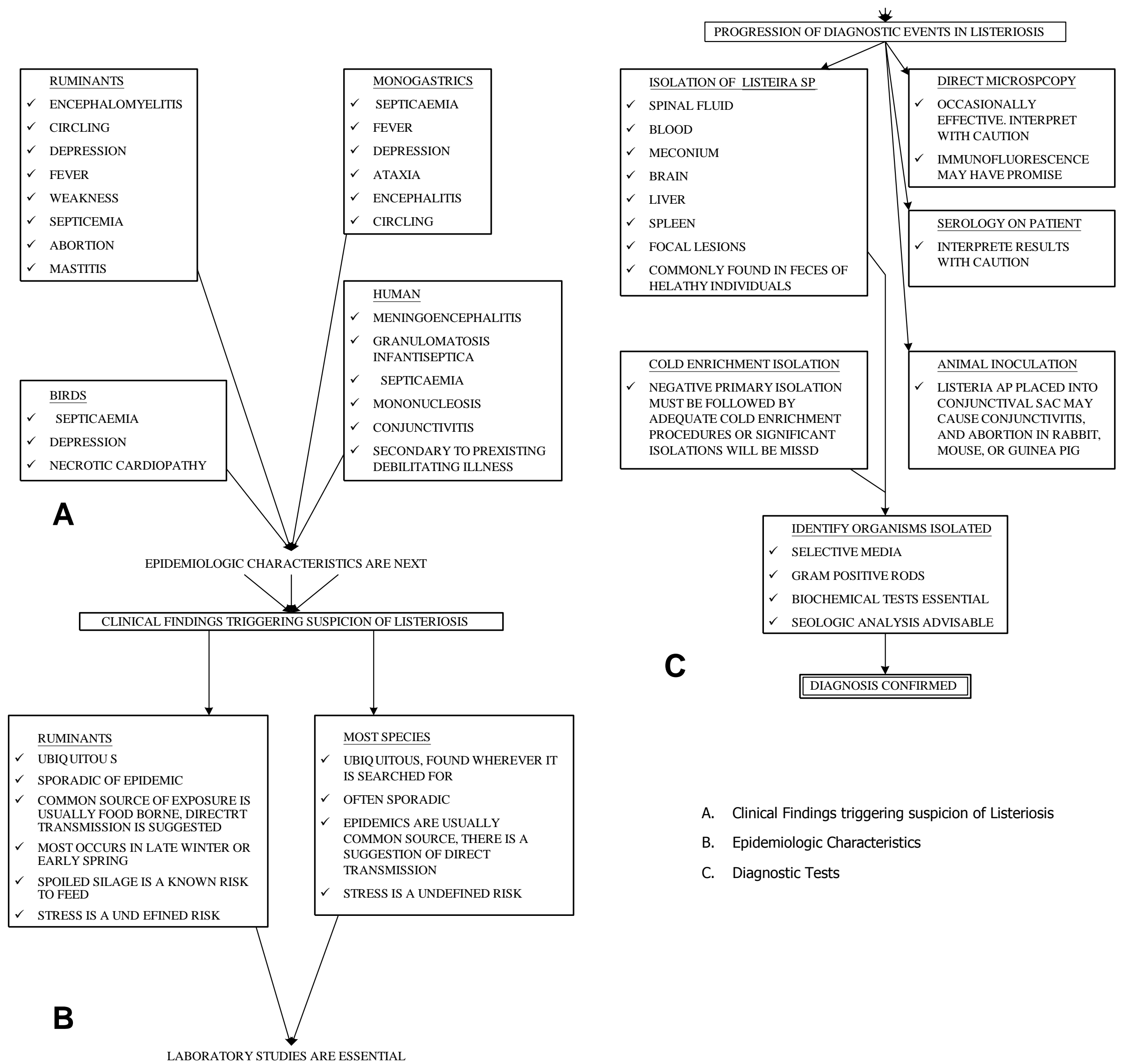
2.15 CLINICAL SIGNS AND SYMPTOMS OF LISTERIOSIS

Clinically, listeriosis is one of the most recently recognized and least understood bacterial infections of man. The clinical signs are not obvious especially in humans. However, a large number of patients with listeriosis often have underlying health conditions which predispose them to listeriosis by interfering with T-cell-mediated immunity. In humans, symptomless faecal carriage is also common of *L. monocytogenes* is common.

The infection starts with sudden onset of fever, headache, nausea and vomiting, and may be followed by meningitis, pneumonia, septicaemia and endocarditis and localized abscesses (Gray and Killinger, 1966; Klinger, 1988). In pregnancy, abortion, stillbirth or premature labour may occur. The infection crosses the placenta and may lead to early-onset or late-onset of neonatal listeriosis in the form of pneumonia, conjunctivitis, meningitis and otitis media (Blenden *et al*., 1987; Schuchat *et al.,* 1991).

Listeriosis in animals is in two forms–the meningoencephalitis and visceral (like the liver, lungs, kidney, spleen and heart). The meningoencephalitic form involves neurological signs with dullness and somnolence. Other signs are droping of saliva and lack of appetite and mastication, lateral deviation of the

head with a tendency of cycling, paralysis with recumbency and death from respiratory failure. The visceral form involves abortion with retained placenta. These presentations are often confused with other disease symptoms like hypocalcaemia, pregnancy toxaemia, gid, meningitis and lopping ill in tick-infested areas (Blenden *et al.,* 1987; Henderson, 1990). The details of some clinical manifestations are shown in Figure 9 segments A and B.



A. Clinical Findings triggering suspicion of Listeriosis
B. Epidemiologic Characteristics
C. Diagnostic Tests

Figure 9: Progression in Diagnostic Events in Listeriosis

Blenden *et al.,* (1987)

2.15.1 Virulence of *Listeria monocytogenes*

The infections and diseases caused by *L. monocytogenes* which is of economic significance have been documented (MacDonald and Carter, 1980; Farber and Peterkin, 1991; Mielke *et al.*, 1993). Reports on the virulence of *L. monocytogenes* have earlier been described (Racz 1969, 1972; MacDonald and Carter, 1980).

Listeriosis 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 immunnodepression or other related conditions 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 (Stelma *et al.*, 1978; Mielke *et al.,* 1993).

The interrelationship of phenotypic and genetic virulence of *L. monocytogenes* is well documented. Phenotypically, the protein P^{60} - 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 P^{60} than the mutants. The rough

(mutants) produced reduced levels of P^{60}, 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 P^{60}, (*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 blow 37°C (Leimeister–Wachter *et al.,* 1992).

Mutants within each of these genes reduce the virulence potential considerably. 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 *prfA*

regulated proteins in *L. monocytogenes* when subjected to such aforementioned conditions (Goebel *et al.,* 1993).

However, the synthesis of catalase, superoxide dismutase enzymes and P^{60} which are all associated with the virulence of *L. monocytogenes* is not under the control of *PrfA* (Goebel *et al.,* 1993). The P^{60} related proteins found as major extracellular proteins in all *Listeria* species are not precursors to virulence, but only P^{60} of *L. monocytogenes* is able to potentiate virulence or restore the failure of R – mutants (exhibiting a drastically reduced synthesis of P^{60}) to adhere to 3T6 mouse fibroblasts. Thus the P^{60} protein of *L. monocytogenes* differs characteristically from P^{60}-related proteins of the non- virulent *Listeria* species (Goebel *et al.,* 1993).

2.16 LABORATORY DIAGNOSIS OF LISTERIOSIS

Culturally, *L. monocytogenes* is readily isolated from blood, cerebrospinal fluid, amniotic fluid, placental tissue, foetal tissue and biopsy materials. These specimens can be directly plated on Oxford *Listeria* selective agar, Palcam agar, Tryptic soy agar containing 5% sheep, horse or rabbit blood and Nalidixic acid blood agar (Blenden *et al*., 1987; Swaminathan and Hayes, 1989)

In practice, isolation, identification and characterization of *L. monocytogenes* from non-sterile specimens like environmental samples relies on cultural procedures involving cold enrichment in a nonselective broth or selective enrichment or both followed by selective plating.Other parameters can be based on morphological, biochemical and physiological characterizations to discriminate *L. monocytogenes* from other *Listeria* species (Seeliger and Hohne, 1979).

Recently with the advent of biotechnology, the conventional microbiological methods are being replaced with more rapid, sensitive and specific methods. Rapid methods or techniques like the polymerase chain reaction (PCR), DNA sequencing, Gene probes, miniaturised biochemical assays, physicochemical assays, antibody – based tests, enzymatic based test and nucleic acid based and pulsed – field gel electrophoresis tests are routinely used for the identification and characterization of *Listeria* species) from clinical, environmental and food samples (Blenden *et al*., 1987; Bubert *et al.,* 1992; 1994; Swaminathan and Feng, 1994; Annukka *et al*., 2005) The details of clinical and diagnostic procedures had been shown in segment C of Figure 9 and laboratory diagnostic procedures in Figure 10.

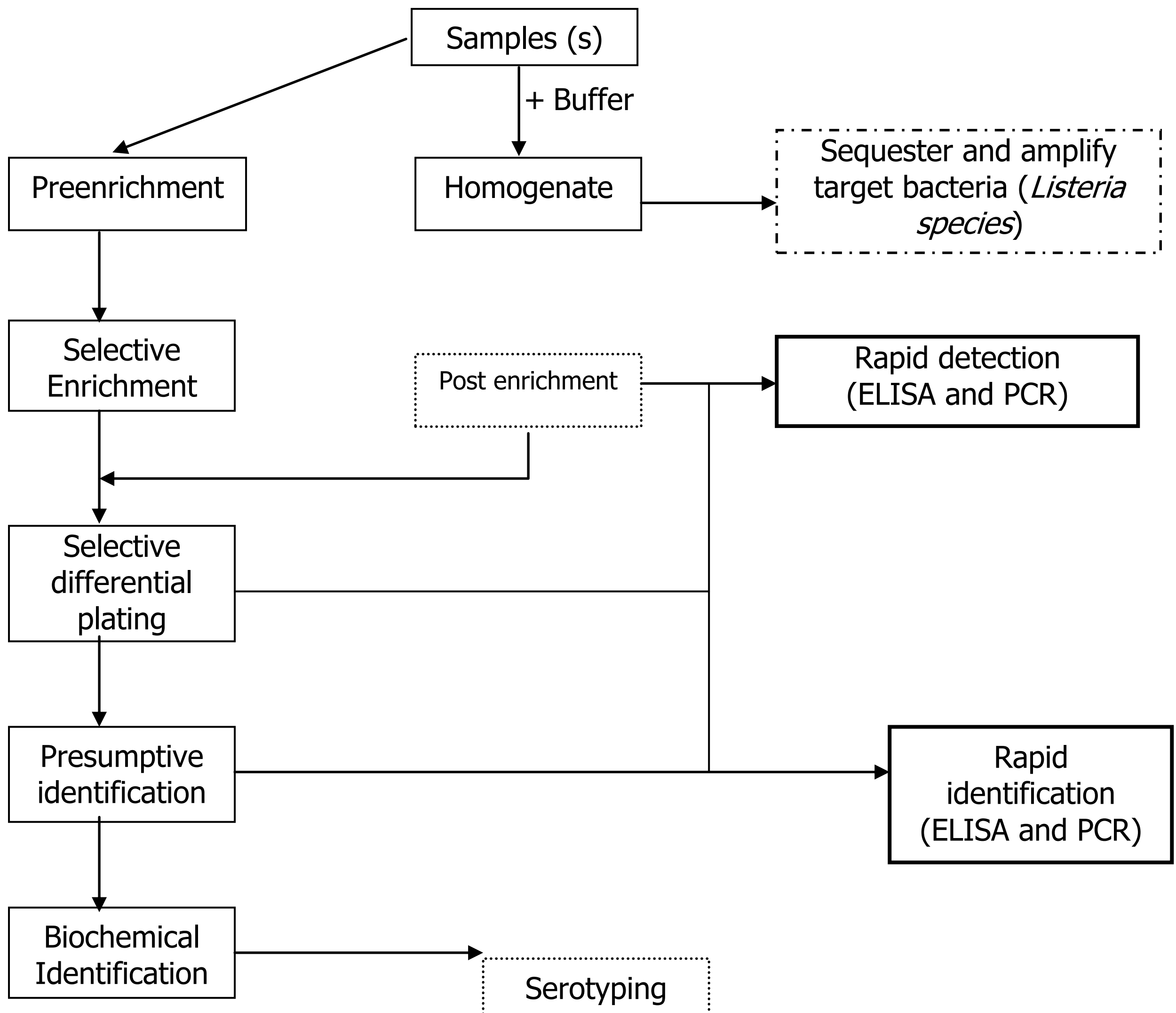


Figure 10: Flow Chart [ℵ] for Cultural, Molecular and Immunological Detection and Identification of *L. monocytogenes* and other *Listeria* species from samples

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[ℵ] Flow chart showing an algorithm for the cultural molecular and immunological isolation and identification of *Listeria* from samples investigated (Solid boxes) conventional culture method (d-shed boxes) optional step; (bold face boxes), rapid method; (dashed – dotted box) future method. (Swaminathan and Feng, 1994)

2.17 CHEMOTHERAPY

Many reports have shown that a high percentage (up to 30%) of patients do succumb to listeriosis even if a deliberate drug regimen has been administered (Neimann and Lober, 1980). However, in occasion where therapeutic measures are stopped too early, an exacerbation has been observed and in addition to some other reasons, the intracellular residence of the organism may account for the resurgence and hence sometimes therapeutic failure (Espage and Reynaud, 1988; Hof, 1991a).

Clinical trials for the treatment of experimental listeriosis have shown that antibiotics like ampicillin are able to reduce the bacterial counts per spleen of a normal mouse without cure until the immune system exerts its beneficial effect When therapy is not started on infection after the bacteria have been established intracellularly, multiplication of the organisms cannot be interrupted completely, as the organism will spread directly from cell to cell without any external stage (Tilney and Portnoy, 1989; Hof, 1991a).

Antibiotic combination have shown that ampicillin /and amoxycillin and gentamycin combination is generally considered as a regimen of primary choice for the treatment of overt listeriosis in man and animals. However, for patients that may be allergic to penicillin, antibiotics of choice are tetracycline, co-trimaxole and erythromycin which nevertheless will not penetrate the CSF. Also, in animals, Coumermycin is the most active antibiotics in the cure of listeriosis in animals. However, the drug cannot be used in humans because of its high toxicity (Hof, 1986; Gellin and Broome, 1989; Hof, 1991b).

The other betalactam antibiotics that have shown higher listeriocidal potency are Mezlocillin, Azlocillin, Gentamycin, Erythromycin, Rifampicin, Tetracycline, Ciprofloxacin and Coumermycin. Erythromycin is often used in place of ampicillin for the treatment of listeriosis in the case of allergy against betalactam antibiotics (Hof, 1986; Gellin and Broome, 1989; Hof, 1991b).

2.18 CONTROL OF *LISTERIA MONOCYTOGENES* AND LISTERIOSIS

The control of zoonotic diseases, knowledge of the epidemiology, the relationship in the infectious factorials as well as important sources and pathways of infections form the basis of combating them. In *Listeria,* studies on infections factorial have revealed the close relationship between the causative agent *L. monocytogenes* and animals and man via food and environment (Fenlon, 1986; Vogt *et al.,* 1990).

Feeds and wastes constitute the primary abiotic factors thus, *L. monocytogenes* can be induced continually via animal feeds especially silage (Fenlon, 1986). Therefore, prevention of animal listeriosis should be based upon the production of ideal silage and waste management. Soiled feeds such as roots or grazing arena which are heavily poached present a risk to the herd. Culling of animals of early diagnosis of the disease may be very necessary for the control of the disease.

Most human infections in urban dwellers and the possible sources of infections include improperly cooked meat, human sewage, sludge, stream, municipal water or rivers and even hospital sickroom's dust (Gray and Killinger, 1966; WHO, 1988). However, steps to prevent the disease are still evolving.

Nevertheless, persons at high risk need to be advised to avoid eating or consuming unpasteurized milk and other milk products, undercooked meat or other food items (Pinner *et al.,* 1992).

CHAPTER THREE

MATERIALS AND METHODS

3.1 AREA OF STUDY

The study was carried out in six Local government areas of the northern part of Plateau State (Figure 11). This region, which has a mean elevation of 1,200metres above Sea level, is cooler than the other Local Government areas and neighbouring Nigerian States. Plateau State is located in the central part of Nigeria and it is divided into seventeen (17) Local Government areas. Six of these local government areas are found in the north while the remaining eleven are within the central and southern Plateau.

The choice of these six Local Government areas was based on the population distribution and agricultural activity. There are large and small agricultural establishments like National Veterinary Research Institute, West Africa Milk Company Vom and other poultry farms within the study area. It was suspected that the unprecedented agricultural activities in the study areas may have influenced the spread of *Listeria species.* The sample collection was carried out throughout the whole year without preference to any season (rainy or dry).

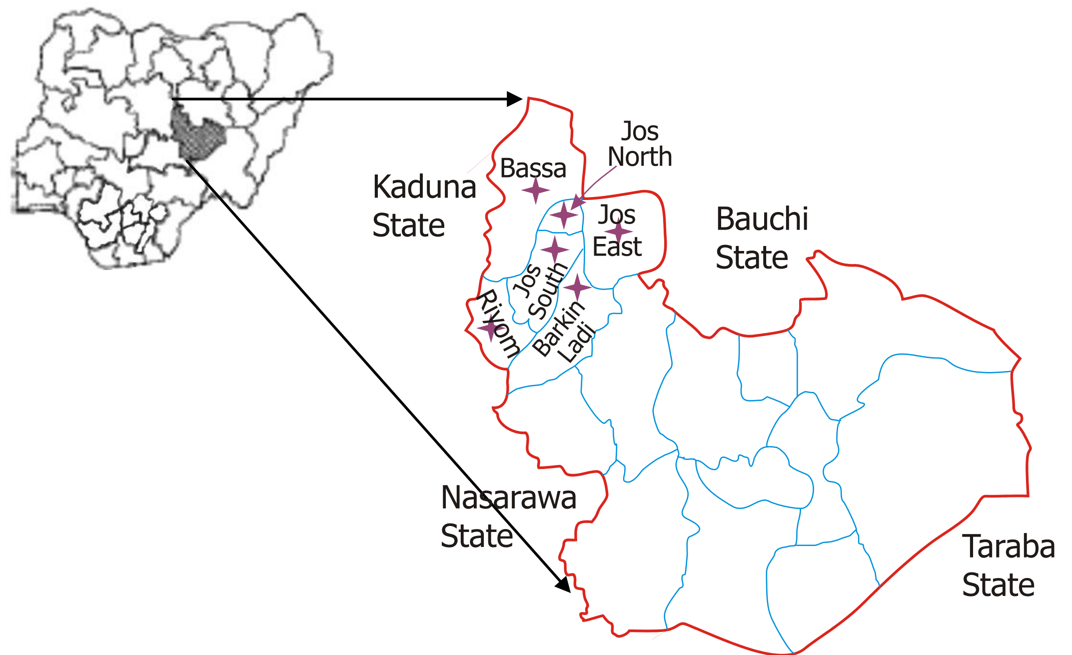


Figure 11: ✦Location of the study areas in Plateau State, Nigeria

(Hopkins, *et al.,* 2002)

Experimental samples were collected at random from fiteen locations/villages where Livestock, poultry, crop and vegetable farms existed for each of the six test local governments. These included agricultural soil samples, vegetation from agricultural lands, farm debris, water samples, livestock faeces (cow, goats, sheep and rabbits), poultry droppings and human faeces.The experimental samples were 10 in number and included: cow dung, farm debris, goat faeces, green vegetation, human faeces, poultry droppings, rabbit faeces sheep faeces, soil samples and water(from mining ponds).

Each sample was replicated 15 times for a particular experimental sample substrate. Five (5g) of each of the ten (10) sample types was collected from each of the fifteen (15) villages (making 150 samples). These samples were collected with the aid of sterile receptacles (rubber gloves) and then placed in sterile plastic bags and sealed with plastic tape.The details of the experimental areas together with the test samples are presented in Table 6.

Table 6: The Experimental Local Government areas and total number of samples collected from them

Local Government Area	Villages	Number of samples
Barkin-Ladi (B/Ladi)	B/Ladi, Sho, Heipang, Kassa, Foron, Rop, Kura-Falls, Dorowa-Babugi, Ganarop, Gashis, Nding, Fan, Razek, Marit and Kuba	150
Bassa	Miango, Rukuba, Kwall, Kpasho, Kake, Mafara, Jebu Miango, Gurum, Jere, Binchi, Gingere, Zagum, Kimakpa, Fuskan-Mata and Mista-Ali	150
Jos East	Angware, Fubur, Lamingo, Shere, Maijuju, Frusum, Maigemu, Gora, Zandi, Fusa, Zarazon, Zigan, Febasduse, Federe and Rizek	150
Jos North	Kabong, Rikkos, Nassarawa, Apata, Angwan Rogo, Naraguta, Jenta Adamu, Utan, Farin Gada, Tudun Wada, Laranto, Angwan Rukuba, Alheri, Gangare, Tafawa Balewa	150
Jos South	Bukuru, Du, Zawan, Zramaganda, Dadin-Kowa, K-Vom, Vwang, Turu, Chakarum, Chugwi, Mai-Adiko (RayField), Gyel, Shen, Hwolshe, Dogon Kafe (Abattoir)	150
Riyom	Riyom, Rahos, Tahos, Rim, Jo’ol, Bajak, Kwi, Kwakwi, Wereng, Sop, Dananse, Bachit, Hawan Kibo, Ganawuri, Asop Falls	150
6	900	900

The samples were immediately transported to the Epidemiology Department laboratory of National Vetrinary Research Institute, Vom, for detailed studies. Samples that could not be subjected to immedidate laboraotory analysis were stored in the refrigerator until they were subsequently analysed for *Listeria* contents. Each of the experimental samples (Table 7 and Plates 1.a-j) was macroscopically examined and cultured in the laboratory for the presence of *Listeria monocytogenes* and other *Listeria* species.

The presumptive isolation, identification and characterization were carried out in Epidemiology Department laboratory of National Vetrinary Research Institute, Vom, while the molecular (genotypic) characterization of some of the *L. monocytogenes* and *Listeria* species isolates into serovars/serotypes was carried out in the Theodor-Boveri-Institut für Biowissenschaften, Lehrstuhl für Mikrobiologie, Universität, An Hubland, 97074, Würzburg, Würzberg, Germany.

However, the pathogenicity test in immunocompromised albino mice and antibiotic susceptibility pattern of *L. monocytogenes* were carried out in Epidemiology Department laboratory of National Vetrinary Research Institute, Vom, Nigeria.

Table 7: Range of Samples Investigated for the Presence of *L. monocytogenes* and other *Listeria* species in the six study areas of Plateau State

Experimental Samples	Study Areas in Plateau State						
	Jos North	Jos South	Jos East	Riyom	Barkin Ladi	Bassa	Total
Cow dung	*15	15	15	15	15	15	90
Farm debris	15	15	15	15	15	15	90
Goat faeces	15	15	15	15	15	15	90
Green vegetation (grazing areas)	15	15	15	15	15	15	90
Human stool	15	15	15	15	15	15	90
Poultry droppings	15	15	15	15	15	15	90
Rabbit faeces	15	15	15	15	15	15	90
Sheep faeces	15	15	15	15	15	15	90
Soil	15	15	15	15	15	15	90
Water (pond)	15	15	15	15	15	15	90
Total	150	150	150	150	150	150	900

* Sample size

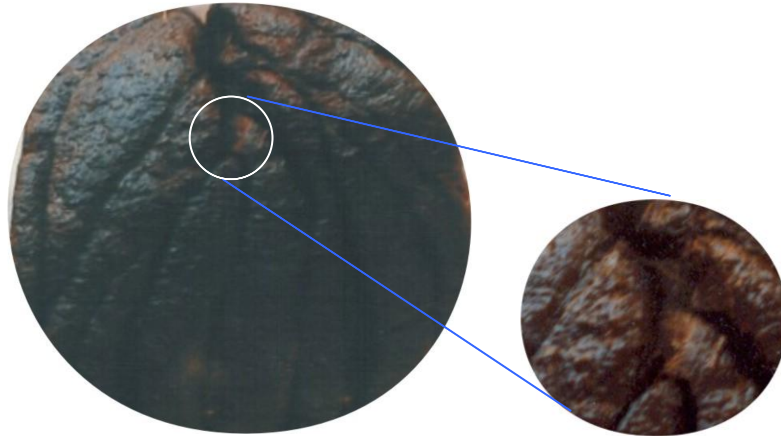


Plate 1.a: Experimental Cattle faecal samples

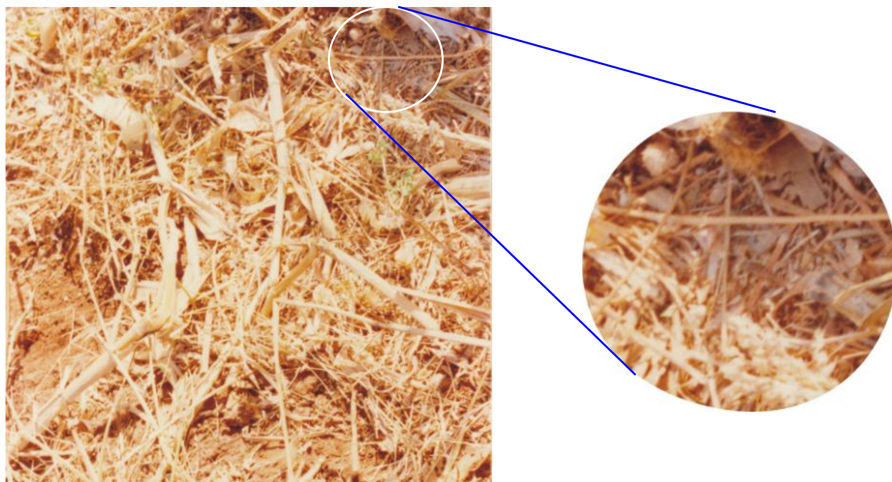


Plate 1.b: Farm Debris employed for the study

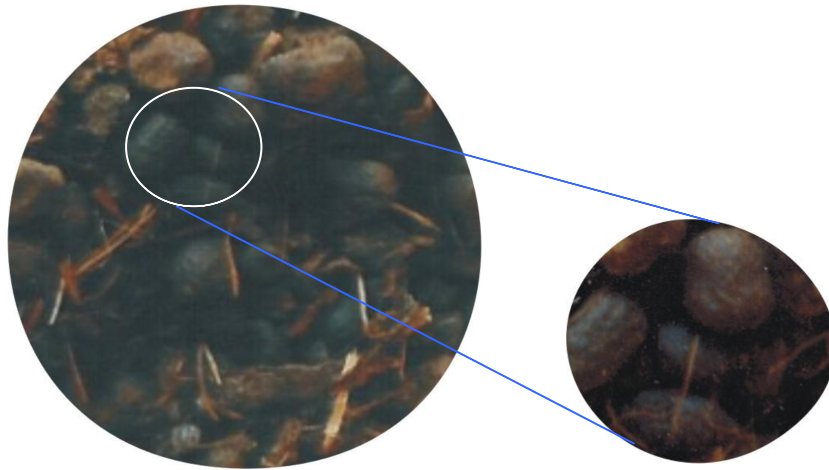


Plate 1.c: Experimental Goat faecal samples

Plate 1.d: Experimental Green vegetation employed for the study

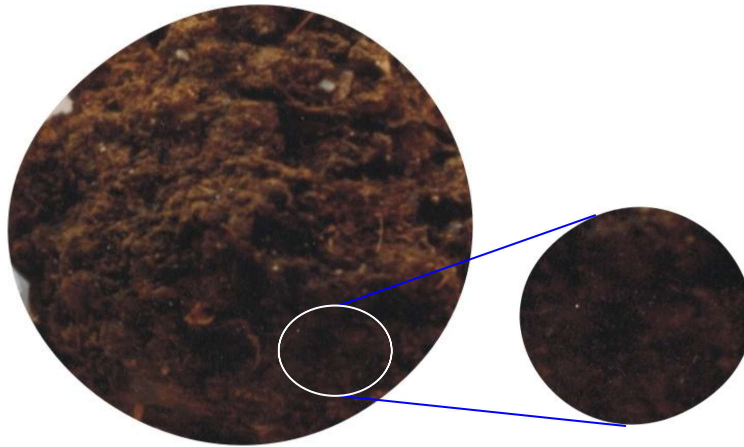


Plate 1.e: Experimental Human faecal samples

Plate 1.f: Experimental Poultry faecal amples

Plate 1.g: Experimental Rabbit faecal samples

Plate 1.h: Experimental Sheep faecal samples

Plate 1.i: Experimental Soil sample

Plate 1.j: Pond Water employed for the *Listeria* study

3.2 LABORATORY ANALYSES OF EXPERIMENTAL SAMPLES

About 10grams of the animal test samples, (cow, goats, poultry, rabbits, and sheep), vegetation, soil, human, farm debris and 200ml volume of pond water were collected from the fifteen different locations according to the villages within the study area.

A total of 900 experimental samples were examined based on the colour, temperature, pH, moisture and dry matter (solid residue for water) content before bacteriological analysis for the presence of *Listeria monocytogenes*. In the laboratory, each sample was divided into two parts. One part was used for the determination of temperature, pH, moisture content and dry matter residues. The other part was assayed for the presence of *Listeria* organisms.

3.2.1 Colour Observation of Experimental Samples

Each sample was observed with the aid of the naked eye macroscopically and the details were recorded.

3.2.2 Determination of Temperature of Experimental Samples

Using the clinical thermometer, the temperature of each experimental sample was taken. The thermometer was immersed into each sample in the receptacle and allowed to stand for two (2) minutes after which the temperature was recorded.

3.2.3 Determination of the pH of Experimental Samples

Each solid experimental sample was pulverized in mortar. A weight of 3g of each pulverized experimental sample was suspended in 100mls of sterile distilled water of pH 7.0. The suspension was then filtered with the aid of

Whatman filter paper (Number 1). The pH meter 450 (conning®) was switched on and allowed to warm for 15minutes. It was then calibrated using standard buffer (pH 7.0).

After the calibration, the pH electrode was cleansed with the aid of absorbent cotton wool and then immersed into the experimental sample filtrate. The pH meter electrode was then allowed to stand in the solution until a sound was heard indicating that a reading had taken place. The pH value of each sample was then recorded.

3.2.4 Determination of Moisture and Dry Matter contents of the Expermental Samples

Approximately, a constant weight of 3g each of solid experimental sample was introduced into a pre-weighed alluminum dish using digital metler balance. The aluminium dishes were placed in a hot air oven and dried at 105°C for a period of 18hrs, after which the dishes were removed and immediately transferred into dessicator jars and then allowed to cool. The dishes were re-weighed and the new weight recorded. The moisture and dry matter contents were calculated and recorded in Table 8 using the formula below.

Moisture content = Weight of dish containing fresh sample – weignt of dish containing dry sample (MC = weight of DC U.S – weight D.C.D.S).

Percentage moisture content = $\frac{\text{weight of moisture}}{\text{weight of sample used}} \times \frac{100}{1}$

(A.O.A.C., 1990)

% Dry matter = $100 - \%\text{moisture content}$

The details of the calculations are given in APPENDIX A.

3.2.5 Determination of Total Solid Residue in Experimental Water Samples

The evaporating dishes which were dried in hot air oven at 100°C for 13minutes were weighed using digital metler balance. A volume of 100mls of each experimental water sample was dispensed into different evaporating dishes (flasks), which were placed in a water bath at 100°C to dry. The dishes were immediately transferred into dessicator jars and allowed to cool.

After cooling, each dish was re-weighed. The new weight of the dish which contained the solid residue was recorded. The weight of the residue was calculated thus:

$$\text{Total solid residue} = \begin{matrix}\text{Weight of dish with residue} \\ \text{after evaporation}\end{matrix} - \begin{matrix}\text{Weight of empty dish} \\ \text{before evaporation}\end{matrix}$$

(A.O.A.C., 1990)

The details of the measurements and calculations recorded are given in APPENDIX A.

3.3 ISOLATION OF *LISTERIA* SPECIES FROM THE EXPERIMENTAL SAMPLES

The second portion of the experimental samples; (Cow dung, Goat faeces, Green vegetation, Human faeces, Poultry droppings, Rabbit faeces, Sheep faeces, Soil and Water) were examined for *Listeria monocytogenes* and other *Listeria* species. This was carried out using standard methods of a two-step enrichment procedure on the samples.

Culture media used in the propagation of the *Listeria* bacteria in the laboratory were prepared as follows. After phenotypic isolation and characterization, the organisms were also genotyped (molecular characterization) by Polymerase Chain Reaction (PCR) method using thermocycler machine.

3.4 CULTURE MEDIA PREPARATION

3.4.1 0.1% Tryptone Soya Broth

Preparation:

One hundred (100) gram of Tryptone soya Broth Oxoid™ CM 129 was weighed and dissolved in 1000mi of distilled water. The solution was thoroughly mixed and distributed 9ml each into macCartney or Universal bottles. The bottles and their contents were then sterilized by autoclaving at 121°C for 15 minutes. After the sterilization, the liquid media were allowed to cool and then, incubated at 37°C for 24hrs to check for sterility and purity of the broth. After the purity test, they were stored at 4°C before use. The details of the chemical composition the TSB are given in APPENDIX A.

3.4.2 Brain Heart Infusion Broth (BHI)

Preparation:

Thirty seven (37) gram of BHI was weighed and dissolved into 1000mi of distilled water.The solution was thoroughly mixed and distributed 9ml each into macCartney or Universal bottles. The bottles and their contents were then sterilized by autoclaving at 121°C for 15 minutes. After the sterilization, the liquid media were allowed to cool and then, incubated at 37°C for 24hrs to check for sterility and purity of the broth. After the purity test, they were stored at 4°C before use. The details of the chemical composition of the BHI are given in APPENDIX A.

3.4.3 University of Vermount (UVM) Modified *Listeria* Enrichment Broth (DIFCO (223-17-2)

Preparation:

Fifty-two (52) gram of UVM was weighed and suspended in 1000mi of distilled or deionized water.The solution was thoroughly mixed and then boiled to dissolve completely and dispensed 9ml each into macCartney or Universal bottles. The bottles and their contents were then sterilized by autoclaving at 121°C for 15 minutes. After the sterilization, the liquid media were allowed to cool and then, incubated at 37°C for 24hrs to check for sterility and purity of the broth. After the purity test, they were stored at 4°C before use. The details of the chemical composition of the UVMSLEB-DIFCO are given in APPENDIX A.

3.4.4 *Listeria* Selective Agar (Oxford Formulation)

Preparation:

Twenty eight (28) gram of the *Listeria* selective Agar base (Oxford formular) CM856 was weighed and suspended in 500ml of distilled or deionized

water. The suspension was gently brought to the boil to dissolve. This was then sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the agar was then allowed to cool to about 50°C. And then, the contents of 1 vial of *Listeria* selective supplement (Oxford formulation) SR140, reconstituted with 5ml of undiluted ethanol/sterile distilled water (1:1) was aseptically added into the molten agar. The molten agar base and the supplement mixture were thoroughly mixed without generation of air bubbles. The agar was aseptically poured into sterile Petri dishes (plates). The plates were allowed to set and then incubated at 37°C for 24hrs for purity and sterility check. After this quality control the plates were stored at 4°C before use. The details of the chemical composition of the *Listeria* selective agar plate are given in APPENDIX A.

3.4.5 PALCAM Selective Agar Base (CM0877)

Preparation:

Thirty five (35) gram of Palcam selective agar was weighed and dissolved into 500mls of distilled water. The content was sterilized by autoclaving at 121°C for 15 minutes. This was allowed to cool to 50°C and then the contents of 1 vial of Palcam supplement (Oxford formulation) SR150, reconstituted with 5ml of sterile distilled water (1:1) was aseptically added into the molten agar.

The molten agar base and the supplement mixture was thoroughly mixed without generation of air bubbles. The mixture was then aseptically poured into sterile Petri dishes (plates). The plates were allowed to set and then incubated at 37°C for 24hrs for purity and sterility check. After this quality control the plates were stored at 4°C before use. The details of the chemical composition of the Palcam the agar plate are given in APPENDIX A.

3.4.6 Nalidixic Acid Sheep Blood Agar

Preparation:

Forty (40) gram of the blood agar base was weighed and dissolved in 10000ml of distilled water and autoclaved at 121°C for 15minutes, allowed to cool to 45°C. And then 20ml of 7% sheep blood and 0.4ml of nalidixic acid solution were added into the molten agar. The molten agar base and the supplement mixture were thoroughly mixed without generation of air bubbles. The mixture was then aseptically poured into sterile Petri dishes (plates). The plates were allowed to set and then incubated at 37°C for 24hrs for purity and sterility check. After this quality control the plates were stored at 4°C before use. The details of the chemical composition of the Nalidixic acid sheep blood agar plate are given in APPENDIX A.

3.5 PROCEDURE FOR THE ISOLATION OF *LISTERIA* SPECIES FROM EXPERIMENTAL SAMPLES

3.5.1 Cow Dung

One (1) gram of each cow dung experimental sample or was pulverized into powder with the aid of surface sterilized pestle and mortar if dry. The powder was then homogenized in 9ml of pre-enrichment broth (0.1% Tryptone soya Broth Oxoid™ CM 129). One part of sample was mixed with nine parts of Brain heart infusion broth (BHI) of pH 7.2. The mixture was allowed to stand on the bench for a period of 6hours to enhance the action of the BHI on the organism. A volume of 1ml of the homogenate of each sample was then inoculated into 9mls of University of Vermount (UVM) Modified Primary *Listeria* Enrichment Broth (DIFCO) and finally incubated at 4°C for a period of 24hours.

After 24hours, a volume of 0.1ml of each sample in the primary *Listeria* selective enrichment broth was inoculated into University of Vermount Modified Secondary *Listeria* Enrichment Broth (UVMSLEB-DIFCO) and then further incubated at a temperature 4°C for a period of one week.

At an interval of 24hours, 48hours and 7days of incubation at 4°C, 0.1ml of each sample in the University of Vermount (UVM) Modified Secondary *Listeria* Enrichment Broth was inoculated in tripplicates on three selective agar plates (Oxford, Palcam and Nalidixic Acid Sheep Blood Agar). The plates were then incubated at 37°C in an incubator in the laboratory for a period of 48hours and examined for typical *Listeria* species colonies and that of other microorganisms (contaminants) after 24 and 48hours incubation.

3.5.2 Other Solid Experimental Samples

For the isolation of *Listeria monocytogenes* and other *Listeria* species each from the other solid experimental samples; (farm debris, Goat faeces, Green vegetation, Human faeces, Poultry droppings, Rabbit faeces Sheep faeces and Soil), they were treated the same as the cow dung.

3.5.3 Water

A volume of 1ml of thoroughly mixed 50mls of each experimental pond water was inoculated in 9ml of pre-enrichment broth (0.1% Tryptone Soy Broth Oxoid™ CM 129) (One part of sample to nine parts of Tryptone Soy Broth) of pH 7.2. The other treatments were the same as carried out on the solid samples for the isolation of *Listeria monocytogenes* and other *Listeria* species.

3.6 PURIFICATION OF *LISTERIA* ISOLATES FOR IDENTIFICATION COMFIRMATION (PHENOTYPIC CHARACTERIZATION)

The purification for *Listeria* species and other microbial isolates started with the pre-enrichment before inoculation into primary, secondary *Listeria* selective enrichment broth and on-sub-culture onto the three different selective agar plates (Oxford, Palcam and Nalidixic Acid). From the solid media, the following procedures macroscopic examination, morphological (Gram reaction), physiological and biochemical reactions enumerated below.

3.6.1 Macroscopic Examination of Culture Plates

Attention was paid to organisms that showed the cultural and evidence of aesculin hydrolysis or black – halo formation on Oxford, Palcam or beta-haemolysis on blood agar plates by the *Listeria* bacteria.

The colonial morphology of the *Listeria species* on the solid media (Oxford *Listeria* agar, Palcam agar and Nalidixic acid agar) were observed after a period of 24, 48 and 72 hours incubation at 37°C. The presumptive identification of *Listeria* species was based on colours, sizes and growth temperatures, colonial morphology (Macroscopic) patterns and the appearance on the solid media compared with reference existing stock cultures of *Listeria* species.

However, other microorganisms that grew on the agar plates were also identified and characterized according to Barrow, *et al.,* (1993). The details of presumptive identification of *Listeria* species on the culture plates were recorded accordingly.

3.6.2 Confirmation Tests for *Listeria* species Isolates

The macroscopic, microscopic morphological features, biochemical and physiological examinations of the *Listeria* cultures were followed by further subjected to the following confirmatory tests. The tests were Gram stain reaction, Beta-haemolysis reaction on blood agar, catalase reaction, motility at room temperature, sugar fermentation (Glucose, Rhamnose, Xylose, Lactose, Sucrose and Manitol), Nitrate reduction test, CAMP test reaction and polymerase chain reaction.

3.6.3 Gram's Stain

The following reagents were used for the staining; Crystal violet stain, Lugol's iodine, Acetone-alchol decolourizer and Neutral red. The detailed chemical composition and preparation of the reagents: Crystal violet stain, Lugol's iodine, Acetone-alchol decolourizer and Neutral red. All the reagents were prepared according to manufacturers instructions.The details of the chemical composition of the reagent are given in APPENDIX A.

Test procedure

Suspected *Listeria* bacteria colonies on any of the agar plates were emusified 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 flame of a Burnsen burner. 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 running tap water.

The smear was again covered with Lugol's iodine for another 30 seconds-1minute and washed off with clean tap waterand was rapidly decolourized (few seconds) with acetone-alcohol. This was washed immediately with clean tap water. The smear was then covered with neutral red stain 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 (×100).

Listeria species that appeared in the Gram-stained slides were tentatively confirmed due to their Gram-positive stain morphology like 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.

3.6.4 Beta-haemolysis Reaction Test

Test procedure

Suspected colonies were streaked on ordinary sheep blood agar plates for haemolytic reactions peculiar to *L. monocytogenes* and other *Listeria* species. The resultant haemolytic reactions were recorded.

3.6.5 Catalase Test

Test procedure

A drop of 3% volume of hydrogen peroxide ($H_2O_{2)}$) was placed on a clean greese- free slide. Then a colony of the *Listeria* growth on nutrient agar medium for 18 to 24 hours was placed on the drop of hydrogen preoxide (H_2O_2) using the

edge of another slide. Observation of bubbles and no bubbles were recorded. Reaction = Organism + $H_2O_2 \rightarrow H_2O + O_2\uparrow$.

3.6.6 Motility Test

The optimum growth temperature for *Listeria* is 28°C to 37°C. After Gram's stain and catalase reactions, cells revealing characteristics of *Listeria* were further subjected to motility in motility agar.The details of the chemical compositions of motility agar medium are given in APPENDIX A.

Test procedure

Using straight wire loop suspected colonies were stabbed into the centre of the tube containing motility test medium to a depth of 5mm and incubated at room temperature for 24 hours to one week. The organisms in the tube that showed umbrella shape on the motility test medium near the microaerophllic subsurface of the medium were recorded.

3.6.7 Biochemical (sugar fermentation) Test for *Listeria* species

All suspected *Listeria*- like organisms were tested for their ability to ferment Glucose, Rhamnose, Xylose, Lactose, Sucrose and Manntol.

Preparation:

15g of Andrade Peptone water was weighed and dissolved in 1000ml of distilled water. Then one percent (1%) of each of the (Glucose, Rhamnose, Xylose, Lactose, Susrose and Mannitol) sugars was prepared using the peptone water solution as diluent. The pH of each sugar was adjusted to 7.2 and then dispensed in McCartney and Bijou bottles with the Glucose sugar containing

Durham's tubes in 10ml amount while others were in 3ml. The various sugar solutions were sterilized by free-steaming at 121°C for 15minutes. The sugars were then incubated at 37°C for 24hrs for purity and sterility check. After this quality control the sugars were stored at 4°C before use. The details of the chemical composition of sugars are given in APPENDIX A.

Test procedure

The *Listeria* isolates to be tested were emulsified in sterile peptone water and incubated at 37°C for 2-3 hours. One drop (0.5ml) of each isolate broth was then used to inoculate the sugars further enriched with sterile rabbit serum. After inoculation, the Durham's tubes were completely filled with the broth solution before test incubation. All the tubes inoculated with *Listeria* organisms were incubated at 37°C and examined at an interval of 24hrs, 48hrs, and 72hrs up to 7 days. Fermentation test reactions indicated by colour change and no gas production in Durham's tubes were recorded.

3.6.8 Nitrate Reduction Test for *Listeria* species

Nitrate Reduction Broth

The medium was used at a concentration of 0.9g in every 100ml of distilled water. A composition of Bacto-peptone (Difco) and Potassium nitrate were weighed and dissolved in 1000ml of distilled water. Four millitre (4ml) of the broth each was dispensed into test tubes containing inverted Durham tube vials and autoclaved at 121°C for 15minutes and stored at cool dark place at room temperature. The details of the chemical composition of Nitrate Reduction Broth are given in APPENDIX A.

Nitrate Reduction Test Reagents

Appropriate weight and volumes of Suphnilic acid, distilled water, Glacial acetic acid (solution A) and Dimethyl-alpha-naphylamine, Distilled water and Glacial acetic acid (solution B) were constituted according to the manufacture's guide. The mixture was warmed with frequent shaking until most of the compound was dissolved. The solution was filtered using Seitz filter. After the sterilization, acetic acid was added to the filtrate and stored in the refrigerator (4°C). The details of the chemical composition of solutions A and B of Nitrate Reduction Test Reagents are given in APPENDIX A.

Test Procedure

Three suspected colonies of *Listeria* species were inoculated in nitrate broth and incubated at 37°C for 24hours. Then 0.1ml of solution A was added to 0.25ml of solution B in test tube. The mixture was observed for two minutes for the development of red colour which indicates reduction of nitrate to nitrite. If red colour was not observed after this period, the test was negative indicating that nitrate was not reduced to nitrite. The results were recorded.

3.6.9 Christie-Atkin-Munch-Peterson (CAMP) Test

The reactions in the CAMP test are useful for the differentiation between haemolytic species of *Listeria* and non-haemolytic ones. Moreso, the test detects synergistic reactions of haemolysis or *Listeria* species with the beta (B) toxin of *Staphylococcus aureus* in conjunction with an exofactor produced by *Rhodococcus equi*. There was an enhancement of haemolysis when *L. monocytogenes* was grown next to *S. aureus* but not when grown adjacent to *R. equi.*

Preparation

Blood agar plates were prepared using washed sheep red blood cells according to (Seeliger and Hohne1979). The cells (5% vol/vol) were added to a Brain heart infusion agar and neutral agar. These cells were washed because sheep blood may contain substances inhibitory to staphylococcal haemolysis. All the agar plates were quality controlled by incubating at 37°C for 24hours. Plates without surface or subsurface contamination were used for the CAMP test reaction.

Procedure of CAMP Test

A plate was inoculated with single streaks of a beta-toxin producing *Staphylococcus intermedius* strains (ATCC49052), *Rhodococcus equi* (ATCC 6939) and *Stapylococcus intermedius* were obtained from Theodor-Boveri-Institut für Biowissenschaften, Lehrstuhl für Mikrobiologie, Universität An Hubland, 97074, Würzburg, Würzberg, Germany.

Stapylococcus intermedius was inoculated in the left vertical streak. *Rhodococcus equi* is inoculated in the right vertical streak. *L. monocytogenes* was on the top horizontal streak showing increased lysis with the *S. intermedius* and *R. equi*. *L. seeligeri* was in the centre horizontal streak showing increased lysis with *S. intermedius* on the left vertical streak but not with *R. equi* on the right vertical streak. *L. ivanovii* below on 3rd horizontal streak shows increased lysis with *R. equi* in the right vertical streak but not with intermedius in the left vertical streak.

The central vertical streak is *S. aureus* which showed increased lysis with *L. ivanovii* than *L. monocytogenes* and *L. seeligeri.* The inoculated plates were incubated at 37°C for 48hrs and examined for zones of haemolysis as a result of beta toxin production.

3.7 OTHER MICROBIAL ISOLATES FROM THE EXPERIMENTAL SAMPLES

Gram-negative bacteria were completely inhibited. However, some unwanted Gram- positive bacteria, fungi and yeasts that were not inhibited were identified by their reactions using gram staining, Lactophenol cotton blue stain, catalase reaction, their non-motility, spore - formation, non-haemolysis and germ tube test reactions only. The organisms included *Staphylococcus* aureus, *Brochothrix* species, *Kurthia* species, *Streptococcus* species, *Mucor*, *Candida albicans*, *Candida* species, *Penicllium* species and *Aspergillus* species.

3.7.1 Identification and Comfirmation of Other Microbial Isolates

***Staphylococcus aureus* (Slide Coagulase Test)**

Gram- positive cocci organisms that were in cluster form, were emulsified in a loop full of normal saline on greese-free slide and allowed to stand for 30 seconds. This was to observe for auto-agglutination. However, when no auto-agglutination was observed, a loopful of human plasma was put, mixed and observed for agglutination. The sugar fermentation and the slie agglutination of the bacterial suspensions were recorded as positive for *Staphylococcus aureus*.

***Streptococcus* species (Catalase Test)**

Suspected Gram- positive cocci organisms that were in chains, catalase and coagulase test reactions negative and sugar fermentation reactions were identified as *Streptococcus* species.

***Brocthrotrix* species** (Motility and Growth Temperature) Gram- positive short rods organisms that were catalase positive, haemolytic, growth at 4°C but non-motile and no growth at 37°C in addition to the sugar fermentaion were identified as *Brochrotrix* species.

***Kurthia* species**

Suspected Gram- positive short rods, that grew at 4°C and 37°C, motile at room temperature but catalase negative, non-haemolytic reactions and sugar fermentation were identified as *Kurthia* species.

***Bacillus* species**

Gram positive short rods that showed growth at 4°C and 37°C, catalase positive and motility were identified as *Bacillus* species. These followed sugar fermentation reactions.

***Candida albicans* and *Candida* species**

Gram-positive cocobacilli organisms were further subjected to germ tube test reaction.

Germ Tube Test for *Candida* species

0.1ml of each yeast growth in 3mls of 0.1% tryptone broth was inoculated into test tubes containing 1ml volume of horse serum. The tubes were incubated at 37°C. Wet films were prepared after 2hours of incubation.

Observation of budding curved germ development from one pole of some of the yeast cells under the microscope (×40 Objective) were identified as *Candida albicans* and those that did not develop buddings were identified as *Candida* species. These were followed by sugar fermentation.

3.8 MACROSCOPIC AND MICROSCOPIC EXAMINATION OF FUNGAL ISOLATES OBSERVED FROM CULTURE OF EXPERIMENTAL SAMPLES

3.8.1 Slide Preparation of The Fungi

Lactophenol Cotton Blue

Preparation

The appropriate weight and volumes of phenol crystals, Lactic acid Glycerol were dissolved in the Distilled water by a gentle heat warming. After the dissolution, Methylene blue dye was added and mixed. This was labelled and stored at room temperature for use.The details of the chemical composition are shown in APPENDIX A.

Test Procedure

***Aspergillus* species**

Macroscopic examination

The macroscopic observation showed that the growth forms were velvety to flaky surface. The colours were variable, sometimes yellow, black and grey-green.

Microscopy

Slide preparations were made from *Aspergillus* fungal growths was made by teasing a portion of the fungi on a drop lactophenol cotton blue stain. The organism in the stain was then covered with cover slip and examined under the microscope using (×40 Objective). The hyphae were septate. Conidiophores were born laterally on the hyphae. There were non-septate and numerous sterigmata that proceeded from the apical club-shaped swellings (head-shaped fructification organs) seen. Also seen were conidia were borne in chains on the sterigmata. Scorings were made and recorded.

***Penicillium* species**

Macroscopic examination

The macroscopic examination showed that the growth forms had a powdery or velvety surface with blue-green colouration.

Microscopy

Slide preparations were made from *Penicillium* fungal growths was made by teasing a portion of the fungi on a drop lactophenol cotton blue stain. The organism in the stain was then covered with cover slip and examined under the

microscope using (×40 Objective). The hyphae were septate and the conidiophores were born vertically from the hyphae. The conidia were born on the conidiophores in multilink chains like a paint brush. Scorings were made and recorded.

***Mucor* species**

Macroscopic examination

The macroscopic observation showed that the growth formed long-fibred and rough woolly net work of hyphae. The colour was initially white but later changed to grey with numerous black dots.

Microscopy

Slide preparations were made from *Mucor* species growths was made by teasing a portion of the fungi on a drop lactophenol cotton blue stain. The organism in the stain was then covered with cover slip and examined under the microscope using (×40 Objective). The hyphae were thick and non- septate. The sporangiophores were departing laterally from the mycelium. Ramified, spherical end of sporangia filled with spores were also observed.The conidia were eliptical and were in large numbers in the sporangia.

3.9 MOLECULAR CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* AND OTHER *LISTERIA* ISOLATES BY POLYMERASE CHAIN REACTIONS (PCR)

After the presumptive identification and characterization of the *L. monocytogenes* and *Listeria* species isolates have been carried out in Epidemiology Department laboratory of National Vetrinary Research Institute, Vom, the molecular (genotypic) characterization of some of the *L. monocytogenes*

and *Listeria* species isolates into serovars/serotypes using PCR was carried out in the Theodor-Boveri-Institut für Biowissenschaften, Lehrstuhl für Mikrobiologie, Universität, An Hubland, 97074, Würzburg, Würzburg, Germany. However, the pathogenicity test in immunocompromised albino mice and the antibiotic susceptibility pattern of *L. monocytogenes* were carried out in Epidemiology Department laboratory of National Vetrinary Research Institute, Vom, Nigeria. Molecular characterization using PCR for the differentiation of *Listeria monocytogenes* and other *Listeria* species isolated, pathogenicity test and the antibiotic susceptibility pattern in this study are presented.

3.9.1 Preparation of *Listeria* species for Polymerase Chain Reaction

The amplification of the entire DNA genes of whole *Listeria* bacteria and invasive associated protein in the medium were investigated using various oligonucleotide primers of *Listeria* species by polymerase chain reaction to Bubert *et al*., (1992) were adopted in this study.

LysozymeE Solution 120mg/ml in Water

Preparation:

Twelf milligram (12mg) of Lysozyme (Sigma) was weighed into Eppendorf tube containing 100ml of dstilled water. This was allowed to dissolve and stored at -20°C until ready for use. The reagents composition is given in Appendix A.

Proteinase K Solution (1.2mg /ml in water)

Preparation:

One point two grams (1.2g) proteinase K (Sigma) was weighed into Eppendorf tube containing 100ml of distilled water to give 0.012g/ml. This was

allowed to dissolve and stored at -20°C until ready for use. The reagents composition is given in Appendix A.

Buffer for Polymerase Chain Reaction

Preparation:

Twenty five grams (25g) of the buffer constituents of Trisphosphate, Borate and Ethylene-diaminetetra-acetic acid (EDTA) was dissolved in 1,000mls of distilled water and the pH adjusted 8.3. The solution was mixed well and sterilized by filtration using Seitz filter and stored at 4°C. The reagents composition is given in Appendix A.

Lysis Prcedure

Three (3) colonies of *L. monocytogenes* and other *Listeria* species from 24 hours culture at 37°C were suspended in various Eppendorf tubes containing 45µl of water +5µl of 10XPCR buffer giving 50µl with the aid of micro-pipette, then 1µl of lysozyme solution was added. This was incubated at 37°C for 15minutes for the lysozyme to act on the organisms.

At the expiration of the 15minutes, 1µl of proteinase-K solution was then added and incubated at 56°C for 15 minutes. In order to inactivate the action of proteinase-K, the solutions were further incubated at 110°C for 5- 6minutes. The reaction volume of 100µL contained chromosomal DNA or crude bacteria lysate (DNA) (10^8). Then 2.5µg of *Taq* polymerase (Pharmacia) was added.

3.9.2 P^{60} Protein Deoxyribonucleic Acid (DNA) Isolation

Brain Heart Infusion Broth was inoculated with a single colony of each *L. monocytogenes* and other *Listeria* to be identified by PCR grown on blood agar at 37°C were harvested from 2ml of Brain Heart Infusion Broth after overnight incubation period. P^{60} protein-invasive associated protein gene (*iap* gene) isolation was performed in the following order:

— 2mls broth was spun at 3,000rpm for 15minutes and the supernatant decanted.

— A loop full of the sediment was suspended in Eppendorf tube containing 45µl of water and 5µl of 10× PCR buffer.

— 1µl of an enzyme-lyzozyme solution was added and incubated at 37°C for 15 minutes.

— Thereafter, 1µl of an enzyme-proteinase K was added and incubated at 56°C for 15 minutes.

This was further incubated at 110°C for 1 minute to inactivate the action of proteinase K and *L. monocytogenes* cells well broken with more release of *iap* gene DNAs. The chemical composition of the reagents are used of are shown in APPENDIX A.

Buffer for Agarose Gel

Preparation:

Fifteen point five grams (15.5g) of the buffer was dissolved in 1,000mls of distilled water, mixed and sterilized by filtration using Seitz filter and stored at 4°C. The chemical composition of the reagents are used of are shown in APPENDIX A.

4% Agarose Gel

Preparation:

Four grams (4g) of Agarose was dissolved in 100ml of 0.5 Tripple buffered saline (TBS) and autoclaved in microwave oven. The gel was then poured onto a glass of 200mm by 200mm in diametre. This was allowed to cool to room temperature prior to usage. The chemical composition of the reagents are used of are shown in APPENDIX A.

3.9.3 Polymerase Chain Reaction (PCR) Test Procedure

The following reagents were pipetted into Ependorff test tubes; 77.5µl of Water was added, followed by 10.0µl of 10 × buffer, Magnesium Chloride ($MgCl_2$) 6.5µl, Primers mono A 1µl + mono B 1µl dNTP 2µl, Chromosomal DNA 1µl and Taq polymerase 2.5µl.

The contents were mixed very well in each tube (Ependorff), and 50µl were transferred into new tubes in pre-warmed PCR machine.

- The tubes were allowed in the PCR machine for denaturation, hybridization and annealing at 94°C for 45 seconds, 56°C for 30 seconds and 72°C for 1 minute 30 times cycle (about 2hours) in a thermocyler machine.
- At the expiration of 2hrs, 20µl of each PCR product of each tube was pipetted into 5µl of bromophenylene dye and mixed well.
- Then the 25µl was pipetted into the appropriate well on 1% agarose gel Lambda ladder Marker (Newe England Biolabs) was used for fragment size determination.

- Standard 8µl of *L. monocytogenes* EGD DNA obtained from University of Würzburg, was added into a different well (Positive control).
- Another well without *L. monocytogenes* DNA (Negative control) giving rise to wells of eight, eleven and twenty wells.
- The samples were electrophorezed through 4% (weight/volume) agarose gel in 0.5 × Tris-borate-EDTA (4mM Tris, 4.5mM botic acid [pH 8.3] and 1mM Sodium EDTA at 200volts and 14°C in a gene navigator system with a hexagonal electrode (Pharmacia, Upsala, Sweden).
- The pulse times ramped from 1 to 15seconds for 18hours.
- The gels were stained with ethidium bromide for 5 minutes, drained and photographed under ultraviolet transillumination and the various migration molecular weights of the *Listeria* species photographed and recorded.

3.10 PATHOGENICITY (INFECTIVITY) STUDY OF *L. MONOCYTOGENES* 4b ISOLATES IN IMMUNOCOMPROMISED ALBINO MICE

The classical tests for *L. monocytogenes* virulence are the Anton conjunctivitis test in rabbits, inoculation of mice and embryonated eggs. However, immunocompromised mice inoculation using the intra-peritoneal (I.P) route of infection as described by Stelma *et al*., (1987) was adopted in this experiment because of its improved sensitivity.

This experiment was designed in order to determine the infective rate of the *L. monocytogenes* serotype 4b which was isolated during the study in immunocompromised mice (Swiss albino mice).

3.10.1 Preparation of Immunucompromised Abino Mice Before *L. monocytogenes* Challenge

A total number of 20 Swiss albino white mice with average weight of (16-18g) obtained from the National Veterinary Research Institute Vom, were used for this experiment. The animals were observed for 7 days and screened for absence of *Listeria* species through the culturing of their faeces.

After the experimental mice had been subjected to quarantine, Carrageenan (Sigmatype II) was dissolved in distilled water (40mg/ml). A volume of 0.5ml of this was then injected intra-peritoneally into each of the 20 Swiss albino mice.This was 24 hours prior to the inoculation of the test animal with the *L. monocytogenes* 4b.

3.10.2 Phosphate Buffered Saline

Preparation:

Twenty nine point five grams (29.5g) of phosphate buffered saline was weighed and dissolved into 1,000ml distilled water with the pH adjusted to pH 7.2. This was dispensed into 10mls aliquots and sterilized at 121°C for 15minutes and stored at 4°C. The chemical components of the phosphate buffered saline (PBS) reagent are shown in APPENDIX A.

3.10.3 Preparation of *L. monocytogenes* 4b Culture

The *L. monocytogenes* was grown at 35°C for 24 hours in tryptone soya broth (TSB). Then 10mls of the culture broth was transferred into each of 2 test tubes (16 x 125mm diameter for each tube). The tubes were then centrifuged at 1600 rpm for 30 minutes. The supernatant was then discarded and the sediment re-suspended in 1ml phosphate buffered saline (PBS). This suspension contained

approximately 10^{10} *Listeria* bacteria/ml (Stelma *et al.,* 1987). The suspension was then diluted to 10^5 bacteria /ml by equal volume of phosphate buffered saline and bacteria plate count of (25×10^4).

3.10.4 Immunocompromised Animal Challenge

Test Procedure

A load of 0.1ml of known pathogenic *L. monoctogenes* ATCC 15313 in guage 27G×1/2 was inoculated intraperitoneally into 5 carrageenan treated mice with the aid of with the aid of Serico™ 0.5ml sterile syrnge. Similarly, a load of 27G×1/2 0.1ml known non-pathogenic *L. seeligeri* ATCC 35967 was inoculated intraperitoneally into 5 carrageenan treated mice (as the test animals) with the aid of with the aid of Serico™ 0.5ml sterile syrnge and this served as the control.

Similarly, with the aid of Serico™ 0.5ml sterile syrnge a load of 27G×1/2 0.1ml of *L. monocytogenes* 4b isolated in this study was inoculated intraperitoneally into 5 carrageenan treated mice. The carrageenan treated 5 mice controls were inoculated with 0.1ml PBS using Serico™ 0.5ml sterile syrnge, 27G×1/2 and this served as the control for carrageenan without any *Listeria* species.

All the treated animals were kept in clean experimental laboratory animal house, in mice cages, under ambient temperature, low humidity, regular supply of clean tap water and pelleted feed (Wood, 1973). The animals were left for observation for a period of 7 days as described by Stelma *et al.*, (1987). The animals that died were subjected to post mortem, (bacteriology for re-isolation of the experimental *L. monocytogenes* antibiogram and histopathology) examinations.

3.10.5 Treatment of Tissues from Experimental Mice for Histopathology

The liver and lung tissues on necropsy from infected experimental animals fixed in ten (10%) formol saline were processed histopathologically. The tissues were sectioned at 6µm using microtome machine and stained with heamatoxylin and Eosin (H & E), and examined under the microscope by ×40 objective according to Luna, (1968) and the results recorded.

3.10.6 Re-Isolation of *L. monocytogenes* 4b from the Experimental Infected Albino Mice

The liver and lung tissues from the experimental mice were homogenized with the aid of surface sterilized pestle and mortar. The paste was then homogenized in 9ml of pre-enrichment broth (0.1% Tryptone soya Broth Oxoid™ CM 129).

One part of sample was mixed with nine parts of Brain heart infusion broth (BHI) of pH 7.2. The mixture was allowed to stand on the bench for a period of 6hours to enhance the action of the BHI on the organism. After 24hours, a volume of 0.1ml of each sample in the primary *Listeria* selective enrichment broth was inoculated into University of Vermount Modified Secondary *Listeria* Enrichment Broth (UVMSLEB-DIFCO) and then further incubated at a temperature 4°C for48 hours.

At an interval of 24hours and 48hours of incubation at 4°C, 0.1ml of each sample in the University of Vermount (UVM) Modified Secondary *Listeria* Enrichment Broth was inoculated in tripplicates on three selective agar plates (Oxford, Palcam and Nalidixic Acid Sheep Blood Agar).

The plates were then incubated at 37°C in an incubator in the laboratory for a period of 48hours and the experimental *Listeria monocytogenes* was indentified according to (Curtis *et al.*, 1989; Barrow *et al.,* 1993) and recorded.

3.10.7 Antibiotic Susceptibility Test *L. monocytogenes* 4b Isolate

*In-vitr*o antimicrobial sensitivity tests were carried out on *L. monocytogenes* using single discs of Enrofloxacin (5μg) CT0639B, Penicillin (10μg), Keproceryl (10μg) AGT06396, (BayerAG), Neomycin (5μg) CT0661B, Gentamycin (10μg), Oxytetracycline (5μg) CT0089B, Ampicillin (10μg) and Pefloxacin (5μg) CT0061 (Oxoid) on 10% sheep blood agar.

The organism from 24hour culture was sub-cultured into a tryptose broth and incubated for 4 hours at 37°C. The 10% sheep blood agar plates were flooded with the culture broth and allowed to stand for 3minutes. This was to enhance the attachment of the organisms on the plate. The plates were then drained off the excess broth into discarding jar. The plates were dried in the incubator for 3minutes before the antibiotics discs were placed at equidistant and incubated over night at 37°C. The zones of inhibition by the antibiotics on the organism were recorded.

3.11 STATISTICAL ANALYSES

The data generated from the analyses of the various samples and the three different solid culture media were statistically analyzed using Analysis of Variance (ANOVA), The Chi Square (X^2) Test and Duncan's multiple range test at a probability of (p= 0.05 and p= 0.001). (Duncan, 1955; Little and Hills 1978).The details of the statistical analyses are shown accordingly.

CHAPTER FOUR

RESULTS

4.1 PHYSICAL AND CHEMICAL EXAMINATION

On examination of samples for colour, temperature, pH and moisture content there were variable indices. The soil sample had a chocolate colour.The powdered farm debris was brownish/ash/carton in colour. The cattle, goat and sheep faeces were darkgreen in colour while that of rabbit was ash/brownish.The human stool brownish/lemon-green in colour, poultry faeces were whitish/ash colour and the water samples were clear/cloudy/muddy in colour.

The experimental samples had a temperature range of 21.2°C to 17.2°C when they were collected. The pH range of the experimental samples was 8.3 to 5.14. The samples had a percentage moisture content of 99.4 to 2.10. The dry matter content was 97.90 to 00.6. The physical characteristics details of the samples are presented in Table 8.

Table 8: The Physical Characteristics of the Experimental Samples

Samples	Colour	Temperature	pH	Moisture content	Dry Matter
Cow dung	Dark Green	21.2°C	6.95	6.64	38.36
Farm debris	Ash/Brownish & brown	19.2°C	5.14	2.10	97.90
Goat faeces	Black	21.0°C	8.17	16.55	83.45
Green vegetation (grazing areas)	Green	18.7°C	6.90	62.24	37.76
Human stool	Brownish/- Lime green	19.5°C	8.12	62.71	37.29
Poultry droppings	Ash/Brownish	20.0°C	7.76	62.73	37.27
Rabbit faeces	Brownish	21.0°C	8.22	48.37	51.63
Sheep faeces	Dark Green	20.2°C	7.37	38.15	61.85
Soil	Dark /Brownish	18.4°C	8.31	6.14	93.86
Water (pond)	Cloudy/Clear/ Muddy	17.2°C	7.87	99.4	00.6

4.2 ISOLATION AND DIFFERENTIATION OF *LISTERIA* SPECIES

Macroscopic examination of *L. monocytogenes* and other *Listeria* species cultures on Oxford *Listeria* agar showed the colonies that were surrounded by black halos due to the hydrolysis of aesculin indicator component of the medium to aesculitin.

Similarly, on the Palcam agar plates, *Listeria* colonies appeared grey–green. The colonies had black–sunken centres with a black halo against a Cherry red background.

Also, *Listeria* organisms on Nalidixic Acid Sheep blood agar plates showed β-haemolysis on the medium. This medium also inhibited the growth of undesired organisms. Nevertheless *S. aureus* and *Streptococcus* species were not completely inhibited. The details of the diagnostic morphology features typical of *Listeria* species colonial growth on selective agar plates used for their isolation from experimental samples are shown in Plates 2, 3, 4 and Table 9.

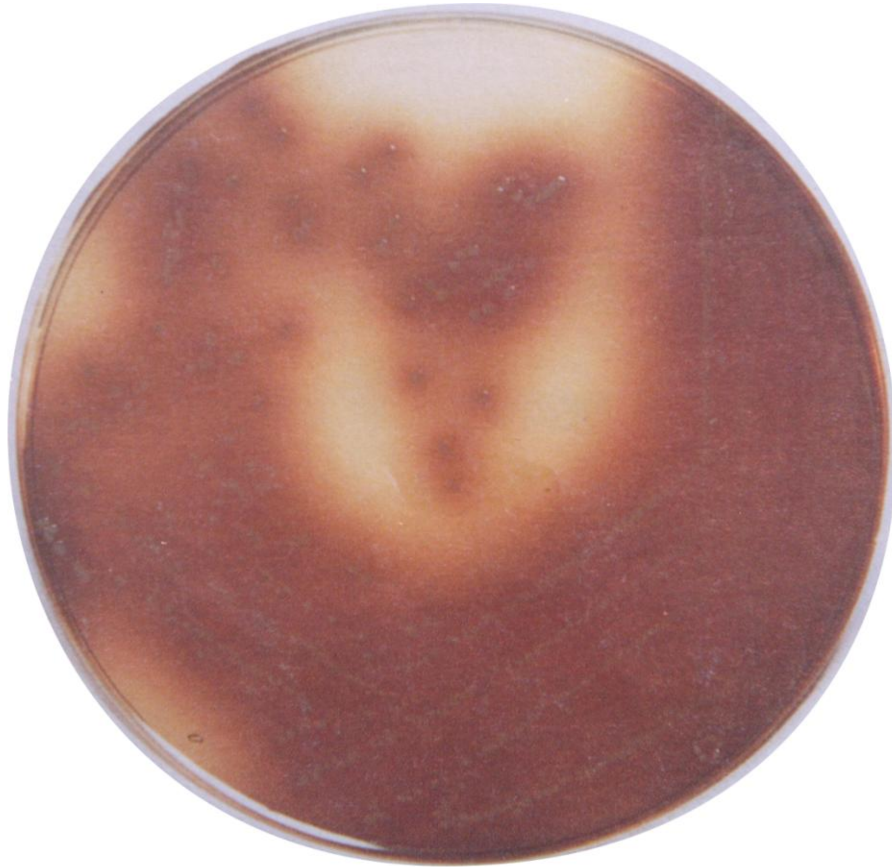


Plate 2: A 48-hour culture of *L. monocytogenes* isolate on Oxford *Listeria* Agar plate showing typical diagnostic features of aesculin hydrolysis to aesculitin (chocolate brown) around the colonies and diffusion into the medium

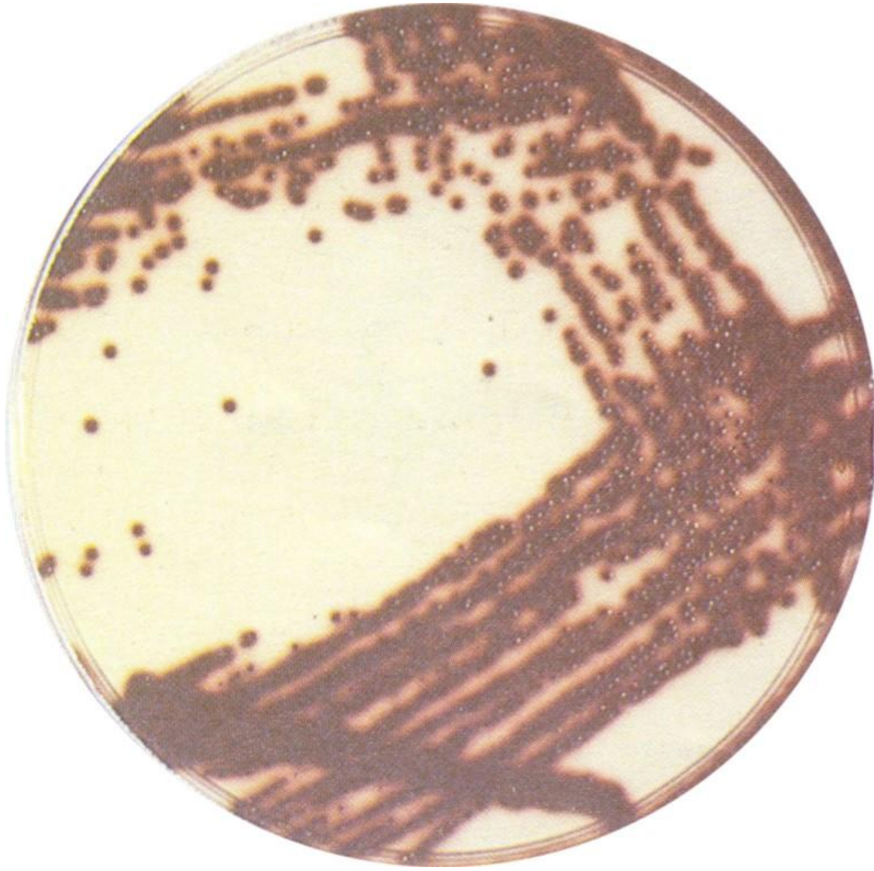


Plate 3: A 48-hour culture of *L. monocytogenes* isolate on Palcam Agar plate showing typical diagnostic features of cherry pigmentations around the colonies and diffusion into the medium

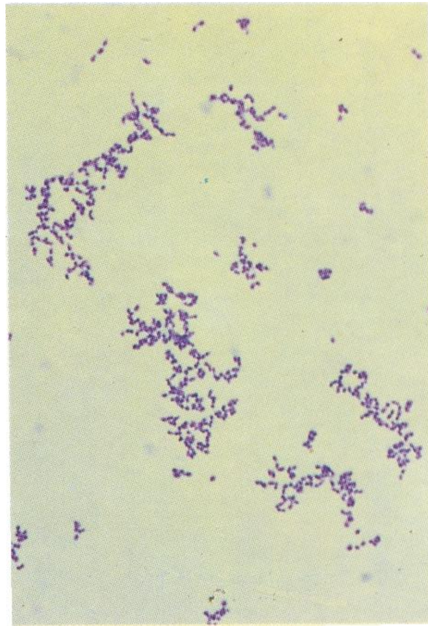
Plate 4: A 48-hour culture of *L. monocytogenes* isolate on Nalidixic Acid Sheep Blood Agar showing typical diagnostic features of zones of β-haemolysis around the colonies in the medium

Table 9: The Biochemical Interaction between the Suspected *Listeria* species and the Growth Culture Media Employed

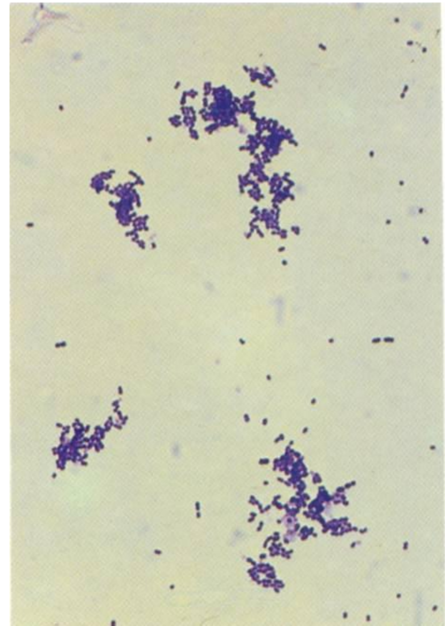
Suspected *Listeria* species	Sources	Media Employed			Growth Temps		Remark
		c/s on OLA	c/s on Palcam	c/s on NABA	37°C	RMT	
1	*CD	Black (1-3mm)	Grey green (1-2 mm)	Cream with β-haemolysis (3mm)	V/-	+	*Listeria* species
3	FD	√	√	√	V/-	+	√
2	GF	√	√	√	V/-	+	√
4	HW	√	√	√	V/-	+	√
8	PD	√	√	√	V/-	+	√
7	RT	√	√	√	V/-	+	√
6	SF	√	√	√	V/-	+	√
5	SL	√	√	√	V/-	+	√
10	Vegt	√	√	√	V/-	+	√
9	WT	√	√	√	V/-	+	√

*CD = Cow dung
+ = motile
C/S = Colour and size
FD = Farm debris
G/vegt = Vegetation
GF = Goat faeces
H/W = Human waste
NABA = Nalidixic Acid Blood Agar
OLA = Oxford *Listeria* Agar
PD = Poultry droppings
RF = Rabbit faeces
RMT = Room Temperature
SF = Sheep faeces
SL = Soil
V/- = Variable or negative
WT = Water
PALCAM = Polymixin acriflavin lithium chloride agar medium
√ = "Ditto" ("Same as above")

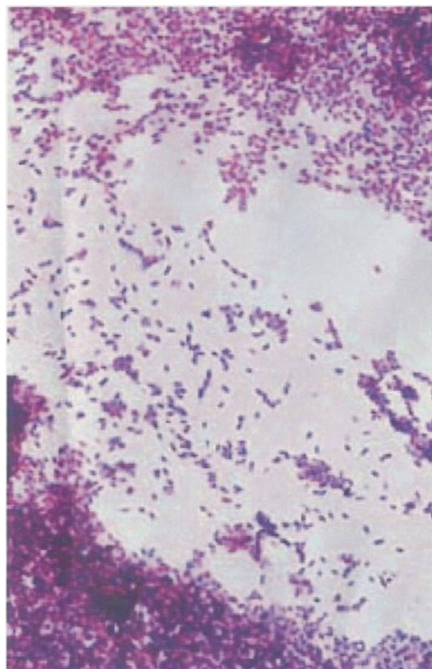
Generally, *L. monocytogenes* and other *Listeria* species were phenotypically differentiation by Gram reaction, beta haemolysis on sheep blood agar, motility, sugar fermentation, nitrate reduction, CAMP test reduction. The details of the resultant reactions of the tests of morphological (Grams reaction) are shown in Plate 5, physiological (beta-haemolysis) (Plate 6), catalase and motility at room temperature Plate 7), biochemical (sugar utilization) (Plate 8), nitrate reaction (Plate 9) and CAMP test reaction (Plate 10), Table 10 and Table 11.



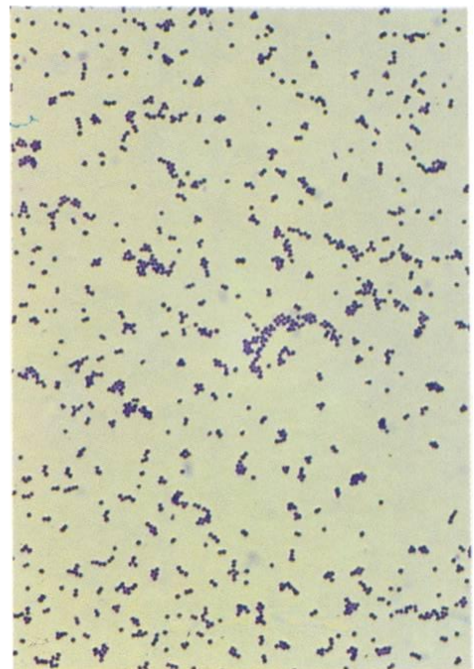
Corynebacterium form
(Chinese Letter)
a



Streptococus form
b



Typical Short rods
c



Cocci form
d

Plate 5: Micrographs oil immersion (×100) representations showing variable Gram- positive forms of *L. monocytogenes* isolated from the samples

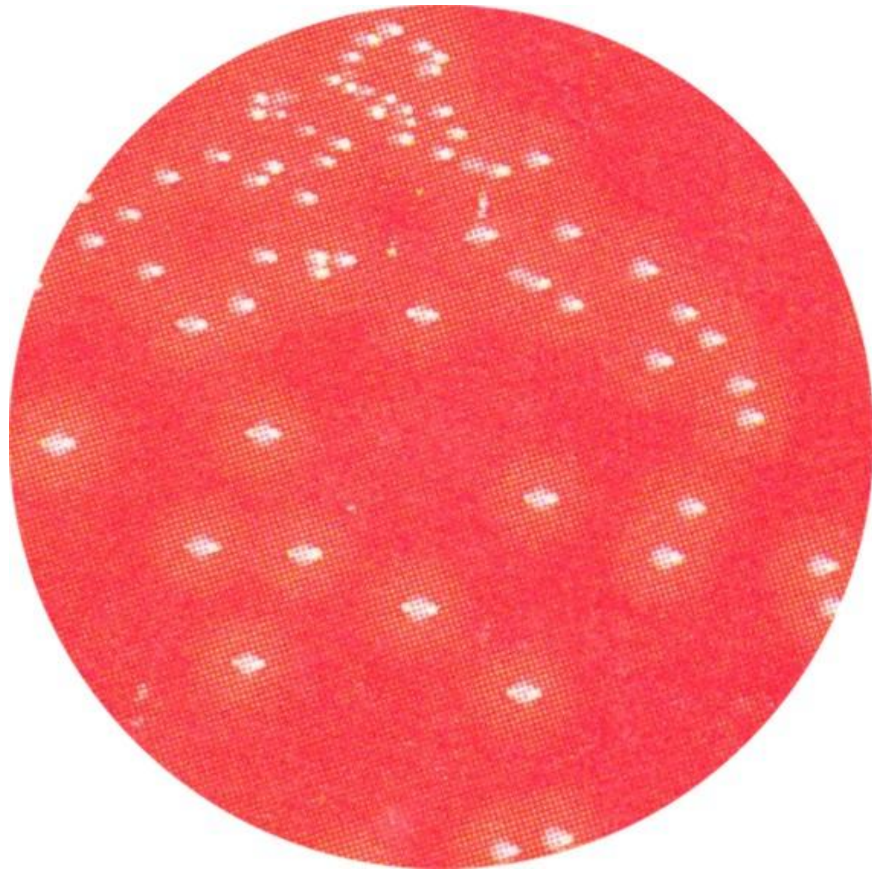


Plate 6: A 48 Hours *L. monocytogenes* colonies showing β-haemolytic Characteristics on Sheep Blood (red bood lyses around the colonies)

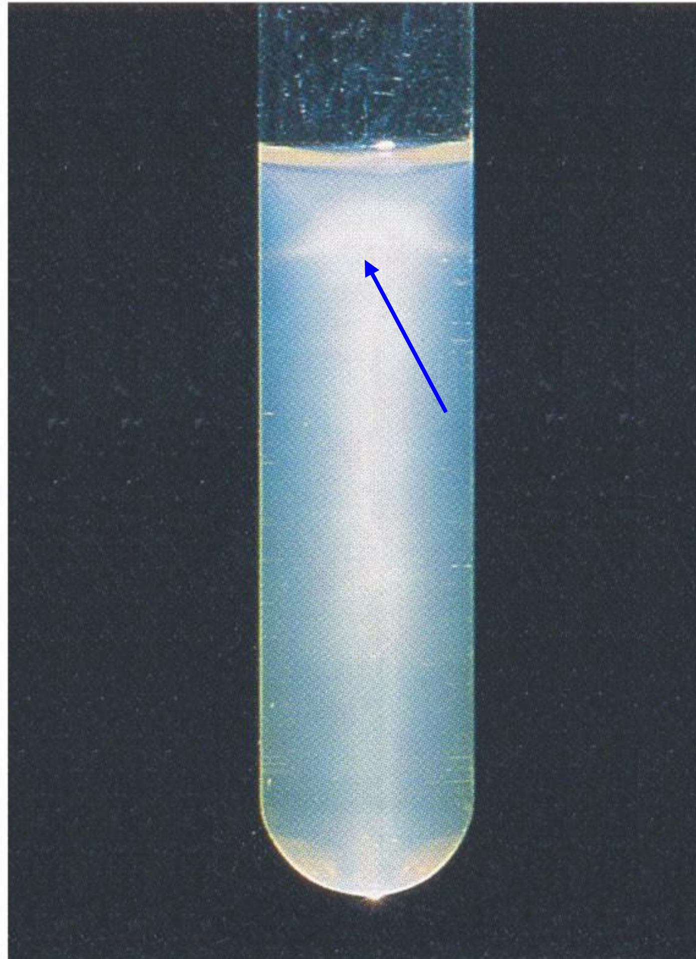


Plate 7: *L. monocytogenes* showing typical umbrella growth characteristic (arrow) in motility medium at room temperature

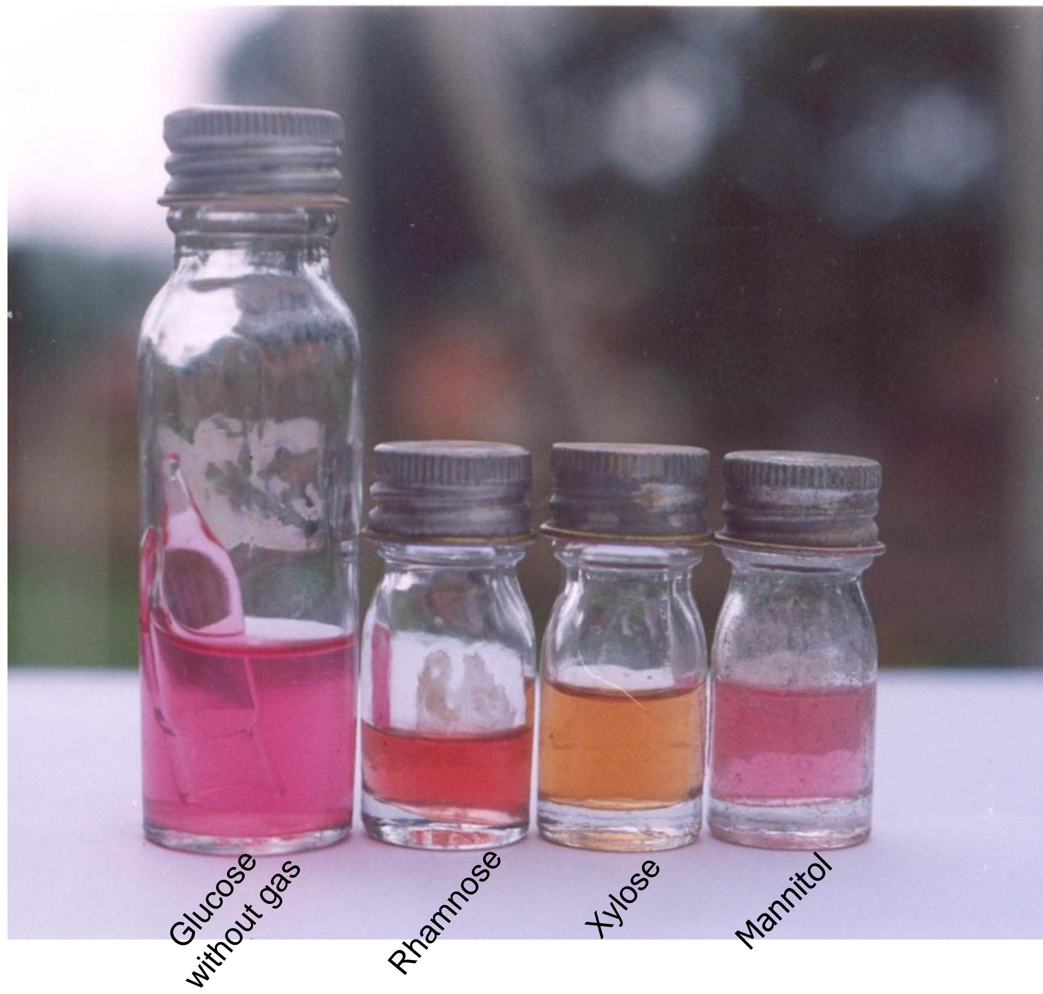


Plate 8: Sugar Reactions of *L. monocytogenes* isolated from the Experimental samples

a. Positive reaction b. Negative reaction

Plate 9: Nitrate Reduction tests of *Listeria monocytognes* isolated from the Experimental samples; a=Positive; b=Negative

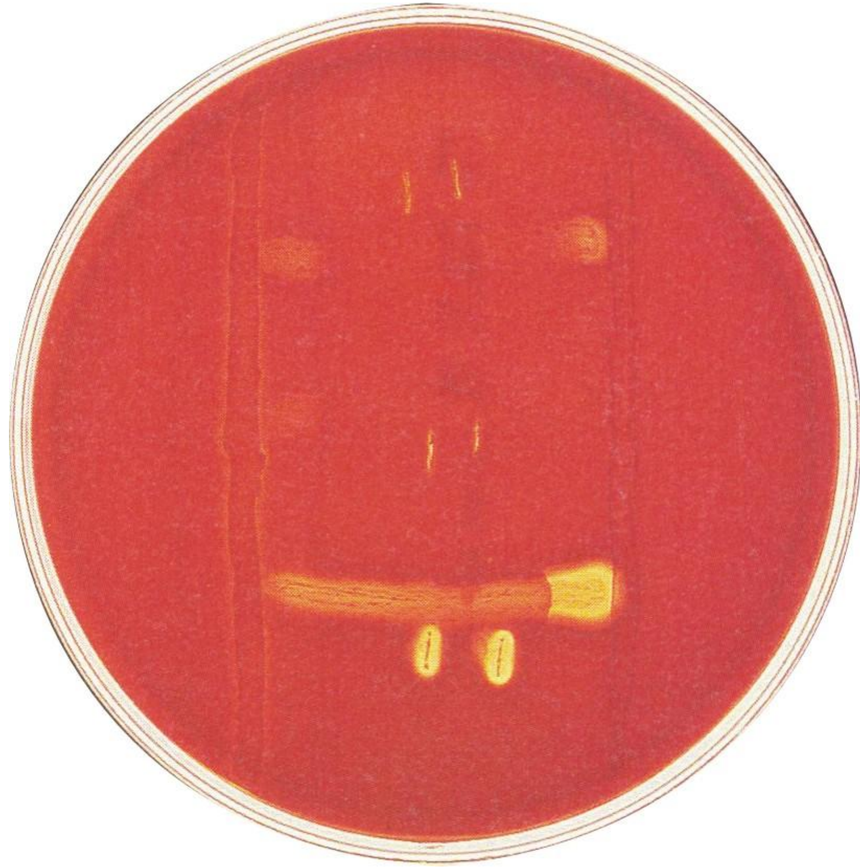


Plate 10: CAMP Test Reaction of *L. monocytogenes* on Nalidixic Acid Sheep Blood Agar showing increased lysis with *S. intermedius but not with R. equi.*

Table 10: CAMP Test Reactions of True *Listeria* species isolated from the Experimental Samples

Listeria species	Haemolytic interaction	
	S. aureus(s)	*R. equi (R)*
L. innocua	-	-
L. ivanovii	-	+
L. monocytogenes	+	-
L. seeligeri	-	-
L. welshimeri	-	-

Table 11: Morphological, Physiological and Biochemical Characterization of *Listeria* Isolates from Experimental Samples

Morphological	Physiological			Biochemical (Sugar Utilization)					Nitrate Reduction Test	CAMP Test		Organism
Gram Reaction	Haemolytic Beta (Sheep blood)	Catalase	Motility at Room Temperature	Glucose	Rhamnose	Xylose	Lactose	Sucrose	Mannitol		*S. aureus(s)*	*R. equi (R)*	*Listeria* specie
+ S*R/CB	+	+	+	+	+	-	√	D	-	-	+	+	*Listeria monocytognes*
+ SR/CB	+	+	+	+	-	+	D	√	-	-	-	+	*Listeria ivanovii*
+ SR/CB	+	+	+	+	-	-	+	+	-	-	-	-	*Listeria seeligeri*
+ SR/CB	-	+	+	+	-	-	+	-	-	-	-	-	*Listeria welshimeri*
+ SR/CB	-	+	+	+	-	-	+	-	-	-	-	-	*Listeria innocua*
+ SR/CB	-	+	+	+	-	-	+	-	+	-	NA	NA	*Listeria grayi*
+ SR/CB	-	+	+	+	-	-	+	-	+	+	NA	NA	*Listeria murrayi*

D = Delayed; SR = Short Rods; CB = Coccobacilli

*+ = Positive; - = Negative; √ = Variable

4.2.1 Occurrence of *Listeria* Isolates Amongst Samples Investigated

Based on the morphological, physiological, biochemical (phenotypic) and genotypic) characteristics, a total of one hundred and eighty-nine (189) isolation of *Listeria* species were made out of the 900 samples screened. Out of the 189(21%) *Listeria* culture positive samples from 900 samples investigated was found to be statistically significant at p = 0.05 and p =0.01, (Table 11). On speciation of the Listeria isolates, *Listeria monocytogenes* was 78 (8.7%), *L. ivanovii* 38(4.2%), *L. grayi* 27 (3%), *L. welshimeri* 16 (1.8%), *L. murrayi* 11 (1.2%) and *L. seeligeri* 6 (0.7%) which were statistically significant at p = 0.05., (Table 12). Also, the various experimental samples showed varying distribution of *Listeria monocytogenes* and other *Listeria* species isolations that were statistically significant at p = 0.05 and p =0.01. The details of the statistical analysis are shown in APPENDIX B.

Table 12: The Distributon of *L. monocytogenes* and other *Listeria* species Isolated from the Experimental Samples

	*CD	FD	GF	G/vegt.	HS	PD	RF	SF	SL	WT	Total
L. grayi	3	4	2	3	3	2	3	1	5	1	27
L. innocua	0	3	0	1	0	0	5	1	2	1	13
L. ivanovii	5	3	4	2	2	5	6	7	3	1	38
L. monocytogenes	10	7	8	7	4	7	11	13	6	5	78
L. murrayi	1	1	0	1	0	1	1	1	4	1	11
L. seeligeri	0	2	0	1	1	1	0	0	1	0	6
L. welshimeri	1	1	1	1	0	2	4	2	1	3	16
TOTAL	20	21	15	16	10	18	30	25	22	12	189

*CD = Cow Dung

FD = Farm Debris

GF = Goat Faeces

G/vegt. = Vegetation

HS = Human Stool

PD = Poultry Droppings

RF = Rabbit Faeces

SF = Sheep Faeces

SL = Soil

WT = Water

The total number of *Listeria* species isolations revealed that *L. monocytogenes* occurred 78 times (8.6%), while *L. ivanovii* was isolated 38 times (4.2%) were statistically significant at $p = 0.05$ and $p = 0.01$. The rate of isolations for the other *Listeria* species is as follows: *L. grayi*, 27 times (3. 0%); *L.welshimeri* 16 times (1.8%); *L. innocua* 13(1.4%); *L. murrayi* 11(1.2%) and *L. seeligeri* 6(0.7%) which were statistically significant at $p = 0.05$ and $p = 0.01$. The details of the results are shown in Figure 12. The details of the statistical analysis are shown in APPENDIX B.

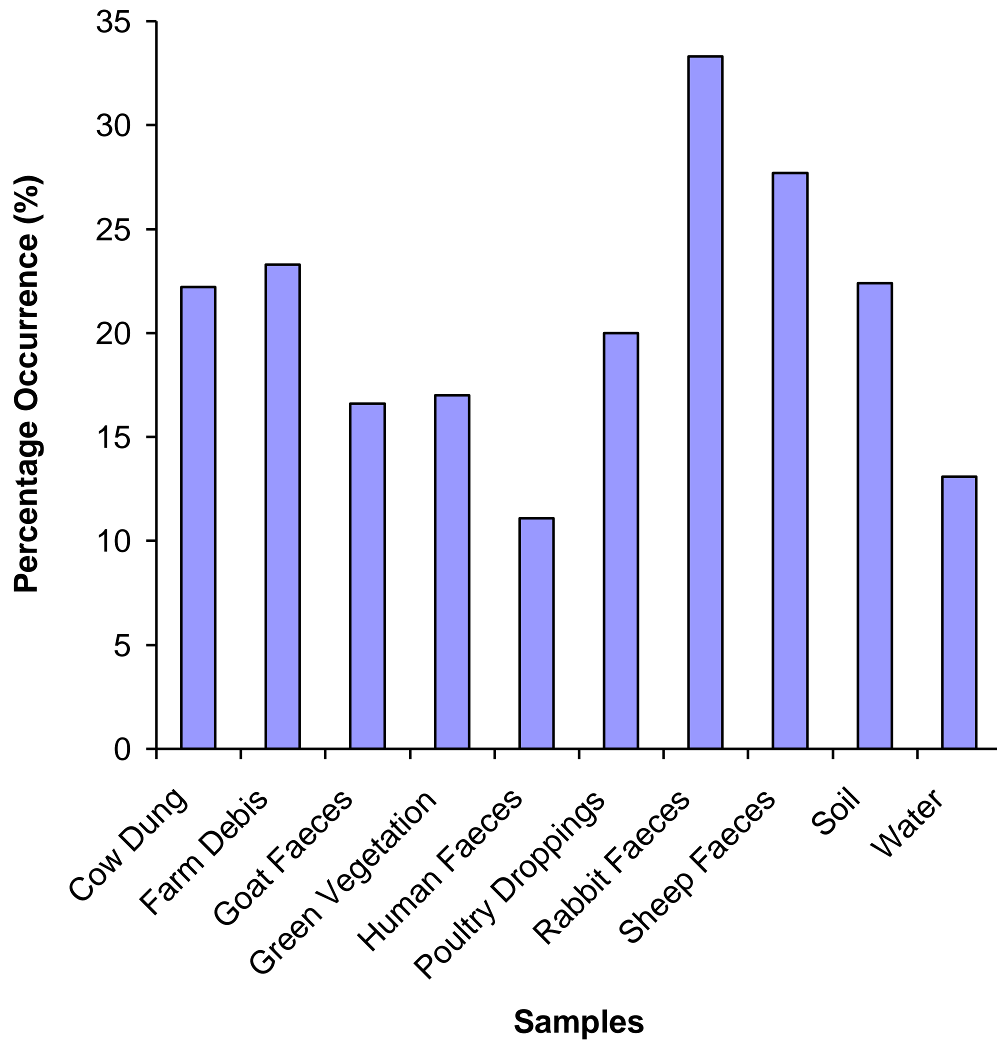


Figure 12: Percentage Occurrence of *Listeria* species among 10 groups each of 90 different experiemental samples Investigated

The results obtained from the different media on the isolation of *Listeria* species revealed that Oxford *Listeria* agar performed best followed by Palcam Agar and then by Sheep Blood agar using Duncan Multiple Range Test, Oxford *Listeria* Agar and PALCAM were not statistically significant at p = 0.05 and p =0.01. However, the isoloation rate of *Listeria* organisms between Oxford and PALCAM when compared with Sheep Blood Agar was observed to be significant at p = 0.05 and p =0.01 using Duncan Multiple Range Test. The details of the results are given in Table 13 and Figure 13.

Table 13: Comparison (Percentage) of the efficiency of the three solid media used in the detection of *Listeria* species

***Listeria* species**	**OLA**	**PALCAM**	**NASBA**
Listeria monocytogenes	78	78	70
Listeria ivinovii	38	27	27
Listeria grayi	27	27	25
Listeria welshimeri	16	16	10
Listeria innocua	13	13	9
Listeria murayi	13	13	9
Listeria seeligeri	6	6	4
TOTAL	189 (100%)	180 (99.5%)	164 (95.5%)

Key:

OLA = Oxford Listeria agar
PALCAM = Polymixin acriflavin lithium chloride agar medium
NASBA = Nalidixic acid sheep blood agar

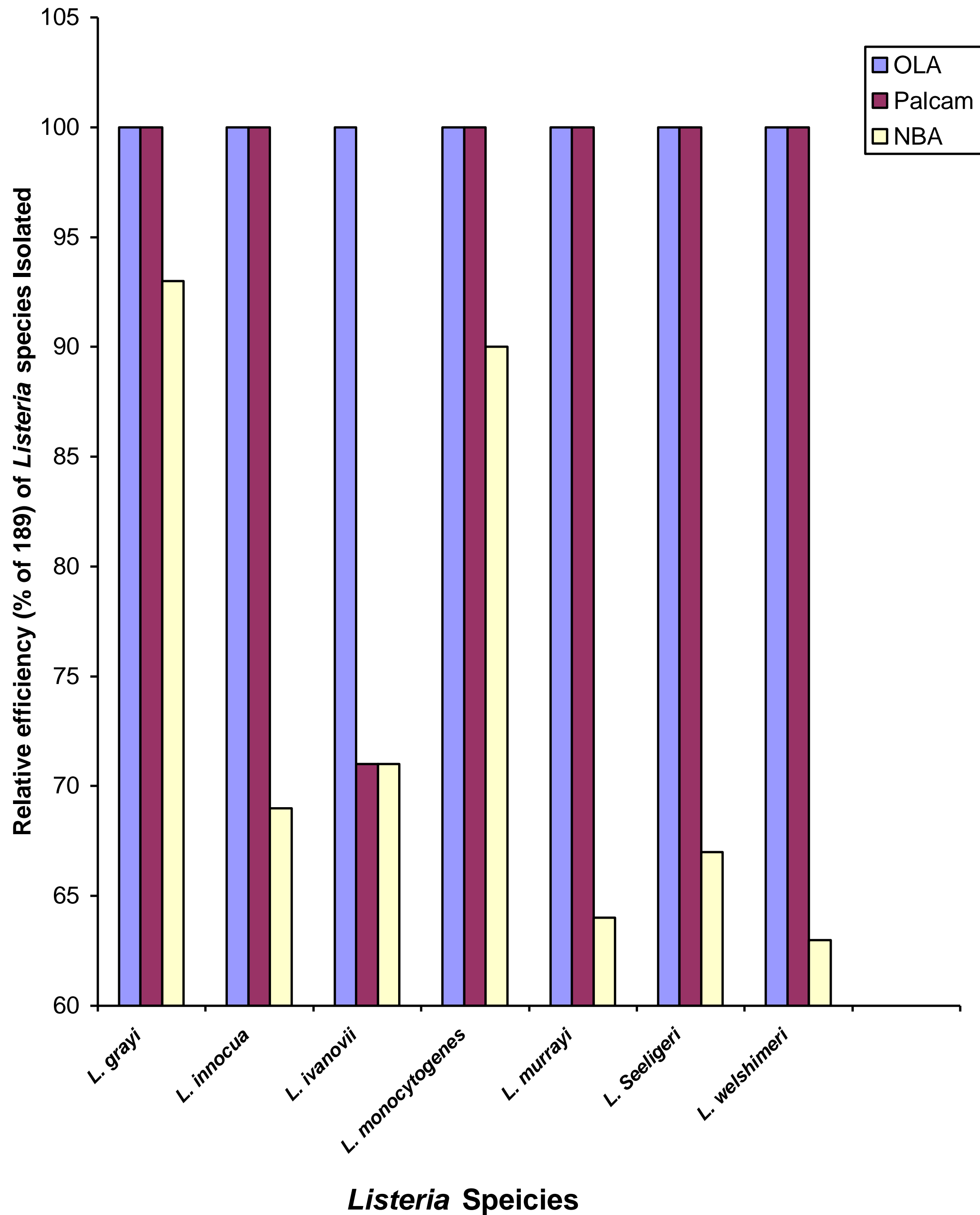


Figure 13: Relative Efficiency of the Three Culture Agar Media Employed in the Detection of the *Listeria monocytogenes* and other *Listeria* species Isolated

Key:
OLA = Oxford *Listeria* agar

NASBA = Nalidixic acid sheep blood agar

Palcam=Polymixin acriflavin lithium chloride agar medium

The details of the statistical analysis are shown in APPENDIX B.

4.2.2 Genotypic Identification of *Listeria* species with the aid of Polymerase Chain Reaction (PCR)

The entire gene from each representative of all *Listeria* species isolated in this study after amplification and analysed in polyacrylamide gel revealed the various sizes of the DNA from the different *Listeria* species isolates. The sizes of the DNA obtained were estimated by their migration in the polyacrylamide gel. *L. innocua* was 1.45Kbp, *L. welshimeri*; *L. seeligeri* and *L. ivanovii* were 1.6kbp. *L. monocytogenes* was 1.5kbp. Similar sizes were obtained for that of *L. grayi* and *L. murrayi*. The details of the results obtained are shown in Plate 11.

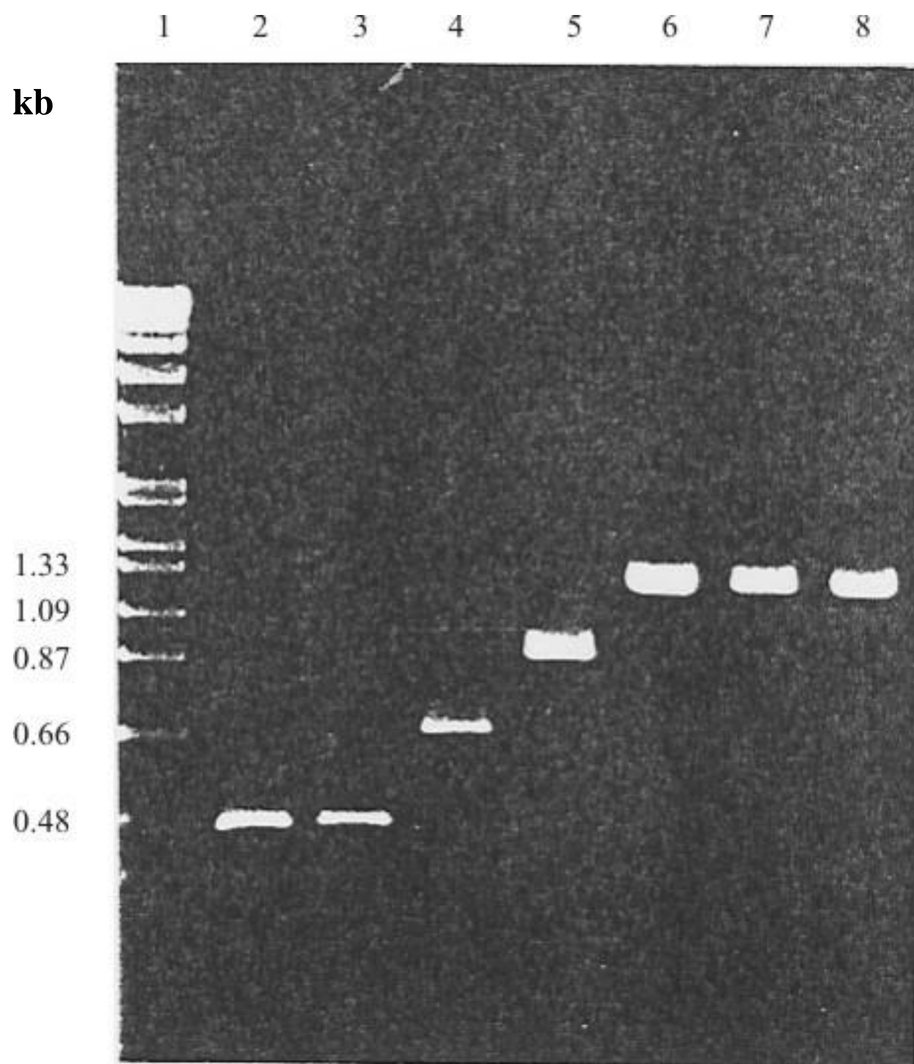


Plate 11: Differentiation of *Listeria* species with a Single PCR Reaction using a 5-Primer Mix (MonoA and MonoB):Lane 1 mol. weight marker; Lane2 *L. grayi*; Lane 3 *L. grayi* sp. *murrayi*; Lane 4 *L. monocytogenes* Sv1/2a; Lane 5 *L. innocua*; Lane 6 *L. ivanovii*; Lane 7 *L. seeligeri*; Lane 8 *L. welshimeri*.

The molecular analysis by Polymerase chain reaction (PCR), on the *L. monocytogenes* and other *Listeria* species isolated from the various samples revealed that the serovars (Serotypes) were, *L. monocytogenes*: 1/2a, 1/2b, 1/2c, 3a, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. In addition, *L. innocua, L. welshimeri, L. seeligeri, L. ivanovii*, *L. grayi* and *L. grayi* sp *murrayi* were identified. The detailed of various DNAs migration of the *Listeria momcytogenes* and other *Listeria* species are shown in Plate 12.

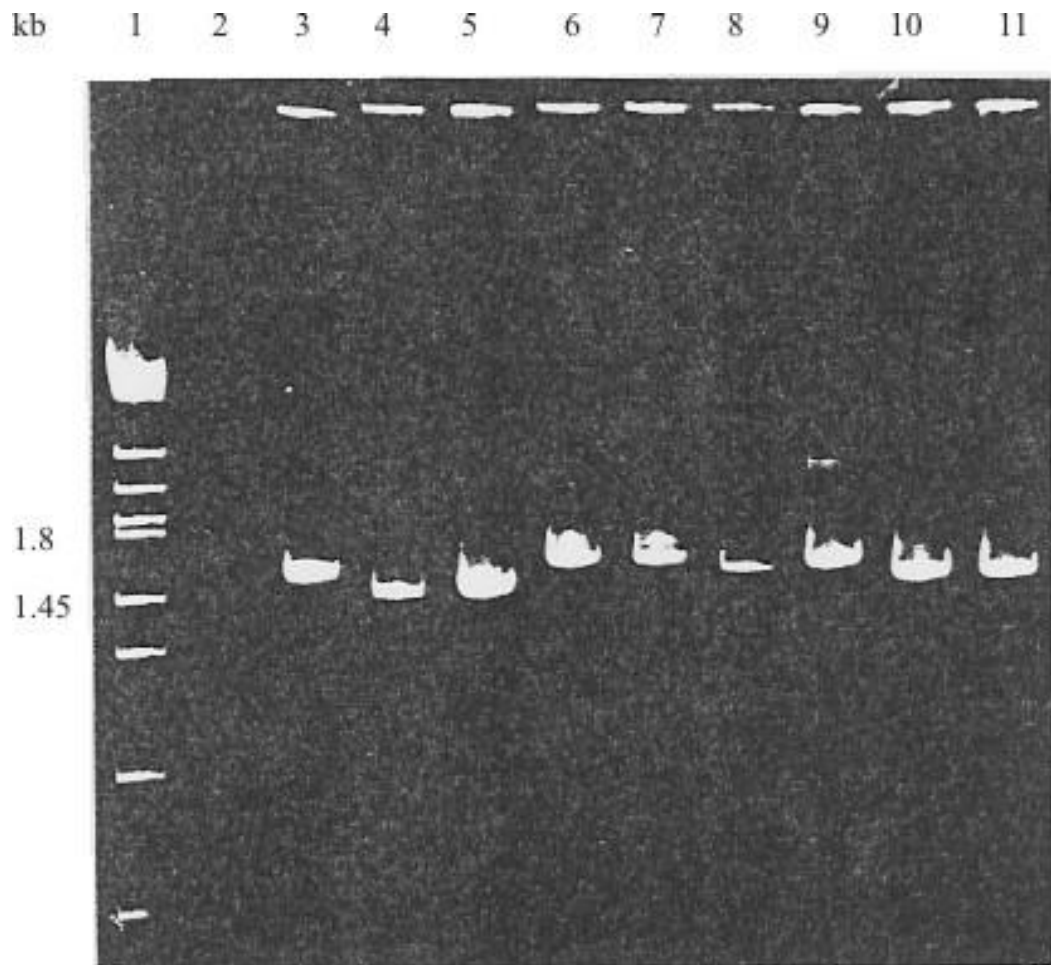


Plate 12: Genus-Specific characterization of *Listeria* species by PCR With oligonucleotide pairs Lis 1A and Lis 1B. The PCR revealed the followings:Lanes 1, *Eco*RI-digested *spp*1 DNA Molecular mass standard; 2, Control Reaction (all reaction ingredients except chromosomal DNA); 3, *L. monocytogenes* Sv1/2a EGD; 4, *L. innocuaSv6a;* 5, *L. innocua Sv6b; 6, L. welshimeri A*; 7, *L. welshimeri* B; 8, *L. seeligeri*; 9, *L. ivanovii*; 10, *L. grayi*; 11, *L. murrayi*

4.2.3 Polymerase Chain Reaction (PCR) Identification of *L. monocytogenes* Strains Using Specific Primers

Fourteen (14) *L. monocytogenes* strains that were characterised into Serovars by PCR products using mono-specific primers (MonoA and MonoB) showed some variations among the *L. monocytogenes*. The nucleotide sequences of invasive associated protein (*iap*) genes deduced from amino acid sequence of P^{60} proteins was used. The detailed molecular migration of various *L. monocytogenes* strains deoxy-ribonucleic acids are shown in Plate 13.

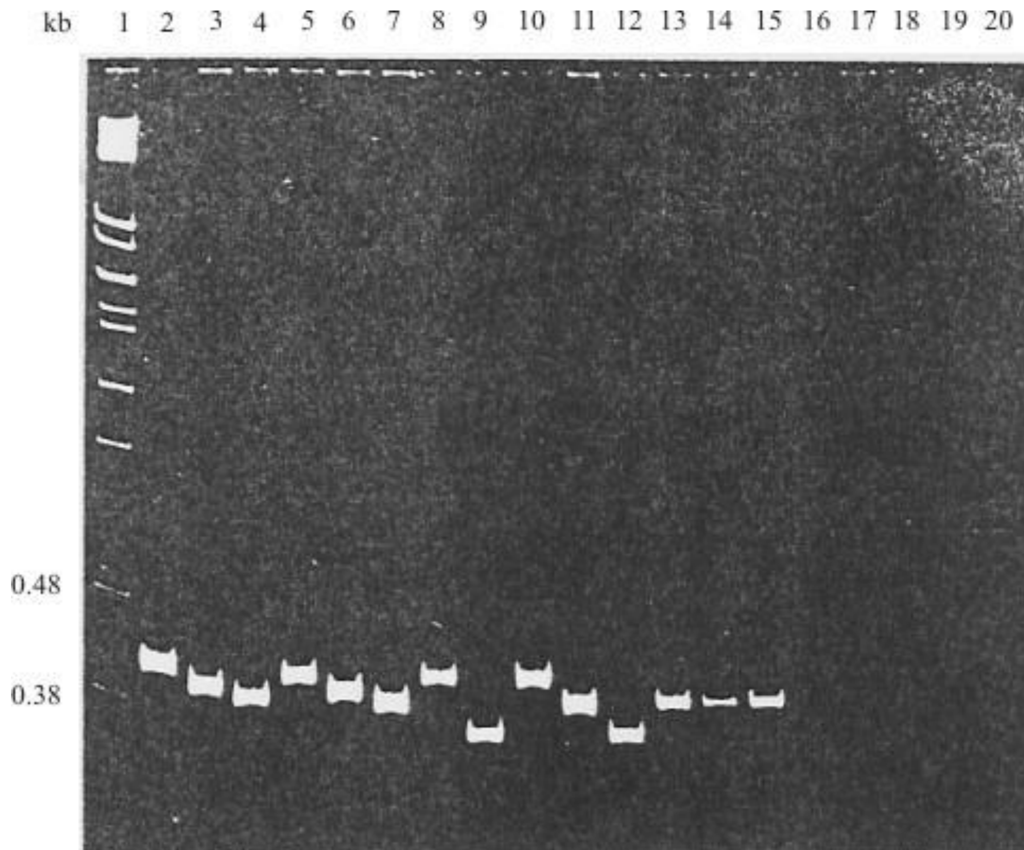


Plate 13: Species Identification of *L. monocytogenes* into serovars by PCR using the primer pair (MonoA and MonoB), Lane 1; mol. weight marker, lane 2; *L. monocytogenes* Sv1/2a EGD, lane 3; *L. monocytogenes* Sv1/2a Mack, lane 4; *L. monocytogenes* Sv1/2b, lane 5; *L. monocytogenes* Sv1/2c, lane 6; *L. monocytogenes* Sv3a, lane 7; *L. monocytogenes* Sv3b, lane 8; *L. monocytogenes* Sv3c, lane 9; *L. monocytogenes* Sv4a, lane 10; *L. monocytogenes* Sv4ab, lane 11; *L. monocytogenes* Sv4b, lane 12; *L. monocytogenes* Sv4c, lane 13; *L. monocytogenes* Sv4d, lane 14; *L. monocytogenes* Sv4e, lane 15; *L. monocytogenes* Sv7, lane 16; *L. innocua, lane* 17*; L. welshimeri*, lane 18; *L. seeligeri*, lane 19; *L. ivanovii*, lane 20; *L. grayi*

4.3 OTHER MICROBIAL ISOLATES

The morphological, biochemical and physiological characteristics of other microorganisms showed that the organisms where *Bacillus* species, *Brochothrix* species, *Kurthia* species, *Staphylococcus aureus*, *Streptococcus* species, *Aspergillus* species, *Mucor* species, *Penicillium* species, *Candida albicans* and *Candida* species. The details are presented in Table 14.

The general distribution of other microorganisms isolated from the various experimental samples; *Bacillus* species, *Brochothrix* species, *Kurthia* species, *Staphylococcus aureus*, *Streptococcus* species, *Aspergillus* species, *Mucor* species, *Penicillium* species, *Candida albicans* and *Candida* species are shown in Table 15.

Table 14: Morphological and Biochemical Characterization of Other Microbial Isolates from Experimental samples

Morphological		Physiological					Biochemical (Sugar Utilization)						Organism
Gram Reaction	Lactophenol Cotton Blue	Coagulase	Haemolytic Beta (Sheep blood)	Germ Tube	Catalase	Motility at Room Temperature	Glucose	Rhamnose	Xylose	Lactose	Sucrose	Mannitol	
+*CC	NA	+	+	NA	+	-	+	-	-	+	+	+	*Staphylococcus aureus*
+CCC	NA	-	+	NA	-	-	+	-	-	+	+	-	*Staphylococcus* species
+SR	NA	-	-	NA	+	+	-	-	-	-	-	-	*Kurthia species*
+SR	NA	NA	+	NA	+	+	+	-	-	-	-	-	*Baccillus* species
+SR	NA	-	+	NA	+	-	-	-	-	-	-	-	*Brochothrix* species
+CB	NA	NA	NA	+	NA	NA	+	-	-	-	-	+	*Candida albicans*
+CB	NA	NA	NA	+	NA	NA	+	-	-	+	-	+	*Candida* species
NA	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	*Aspergillus* species
NA	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	*Penicillium* species
NA	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	*Muccor species*

SR = Short Rods; CB = Coccobacilli; +*CC = Positive Coccii cocci in clusters;
CCC = Positive cocci in chains; CB = Coccobaccilli; - = Negative;
NA = Not Applicable

Table 15: The Distribution of Other Microbial Species Isolated in the Test Samples

Microbial species	Soil	Rabbit Faeces	Farm Debris	Water	Cow Dung	Vegetation	Goat Faeces	Sheep Faeces	Poultry Droppings	Human Faeces	Total
Aspergillus species	3	1	1	-	2	1	-	-	6	2	14
Bacillus species	6	8	3	-	1	5	-	-	5	-	15
Brochothrix species	1	-	-	-	-	-	-	1	1	-	3
Candida albicans	-	3	-	-	3	-	1	1	6	9	20
Candida species	3	2	2	-	2	-	-	1	4	7	19
Kurthia species	2	-	-	-	-	-	-	-	1	-	3
Mucor species	4	5	3	-	3	2	1	1	9	2	23
Penicillin species	4	2	2	-	5	1	2	3	5	1	22
S. aureus	5	7	3	5	6	8	3	4	7	3	31
Streptococcus species	7	3	-	1	2	3	-	-	4	8	21
Total	35	31	14	6	24	20	7	11	48	32	171

(Column headers above sample columns: Occurrence — Test Samples)

4.4 PATHOGENICITY (INFECTIVITY) STUDY OF THE *LISTERIA MONOVYTOGENES* 4b ISOLATE ON IMMUNOCOMPROMISED ALBINO MICE

On infection with *L. monocytogenes* 4b the animals stabilized and were normal for two days without any clinical signs. However, before death, the animals showed signs of diarrhoea, dehydration and anorexia after 48hours post infection and died on the 3rd and 4th days respectively.

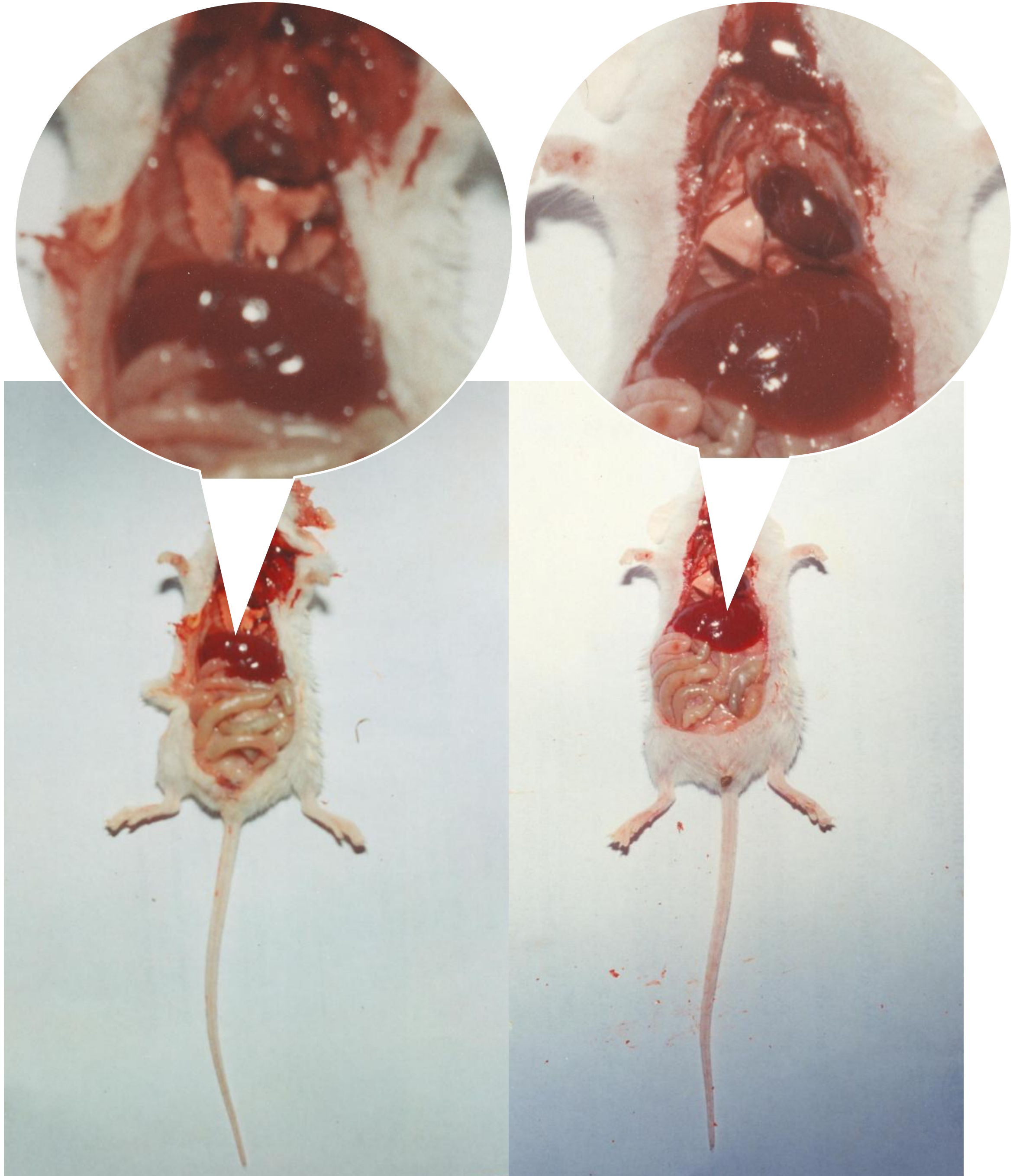
From the results of the pathogenicity study, a hundred percent (100%) death was recorded in the experimental mice. The five (5) positive control mice and the five (5) tested with our *L. monocytogenes* 4b bacteria died within 3 and 4 days post inoculation, while the two sets five (5) negative controls of apathogenic *L.seeligeri* and carrageen were all alive.The details of the results are shown in Table 16.

Table 16: Pathogenicity of the *L. monocytogenes* 4b on Immunocompromised Albino Mice

	Pathogenicity of the *L. monocytogenes* species			
Immunocompromised Albino Mice	Test Mice with Isolate of *L. monocytogenes*	Positive Control with *L. monocytogenes* ATCC15313	Negative Control with *L. seeligeri* ATCC35967	Carrageenan Negative Control (PBS)
Number Inoculated	5	5	5	5
Casualties	5	5	—	—
Number of Survival	—	—	5	5

4.4.1 Post Mortem Examination of the Infected Abino Mice

On necropsy, the gross pathology showed inflamed ileum with marked oedema of the Caecal wall with necrosis of the mucosa. Also, two of the predilating organs – Liver and lung tissues contained numerous pinpoint necrotic foci and congested appearance. The details of the gross pathology results are shown on Plate 14.



a. Infected with test organism (*L. monocytogenes*)

b. Infected with PBS

Plate 14: Gross Pathology of the infected Mouse (a) and the non-infected Mouse (b).The infected experimental mouse showed haemorrhagic and congested lungs, necrotic liver foci and also liver pin-point haemorrhage

4.4.2 Histological Examination of Infected Tissus of the Abino Mice

The histopathology analysis carried out on infected liver and lung samples revealed that liver cells infected by *L. monocytogenes* exhibited some peculiarities. The liver blood vessels showed severe congestion multifocal pale areas that were caused by the presence of *L. monocytogenes*.The liver also showed aggregates of vacuolated hepatocytes (hepatocellular necrosis) with dark stained short - rod shaped organisms, (*L. monocytogenes).* This elicited severe infiltration of polymophonuclear cells within and around the walls of the blood vessels and completely eroded blood vessel walls. Some of the vessels exhibited signs of fibroplasias.

The lungs showed signs of severe haemorrhage within the interlobular septa. All cells of the interstitial stroma were observed to be undergoing karryorrhesis. The walls of the capillary vessels were found to have been thickened with signs of severe fibroplasia around the bronchial wall. The details of the results are presented in Plate 15 and Plate 16.

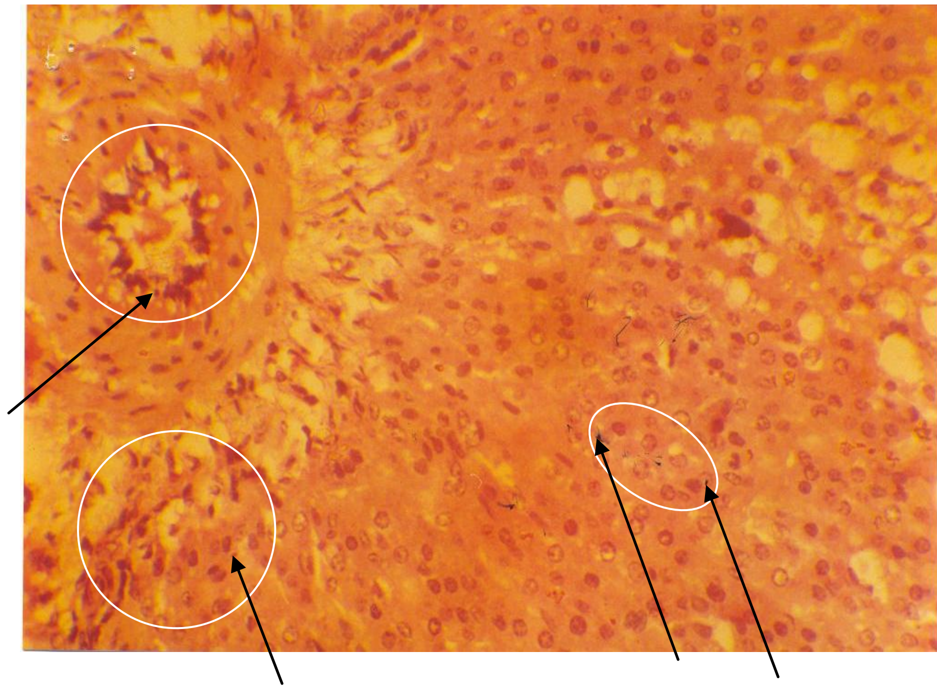


Plate 15: Slide micrograph of the liver tissue of the infected mouse stained with H & E showing short rods *(L. monocytogenes*), congestion of blood vessels with vacuolation of haepatocytes and proliferation of fibrogblast around the blood vessels

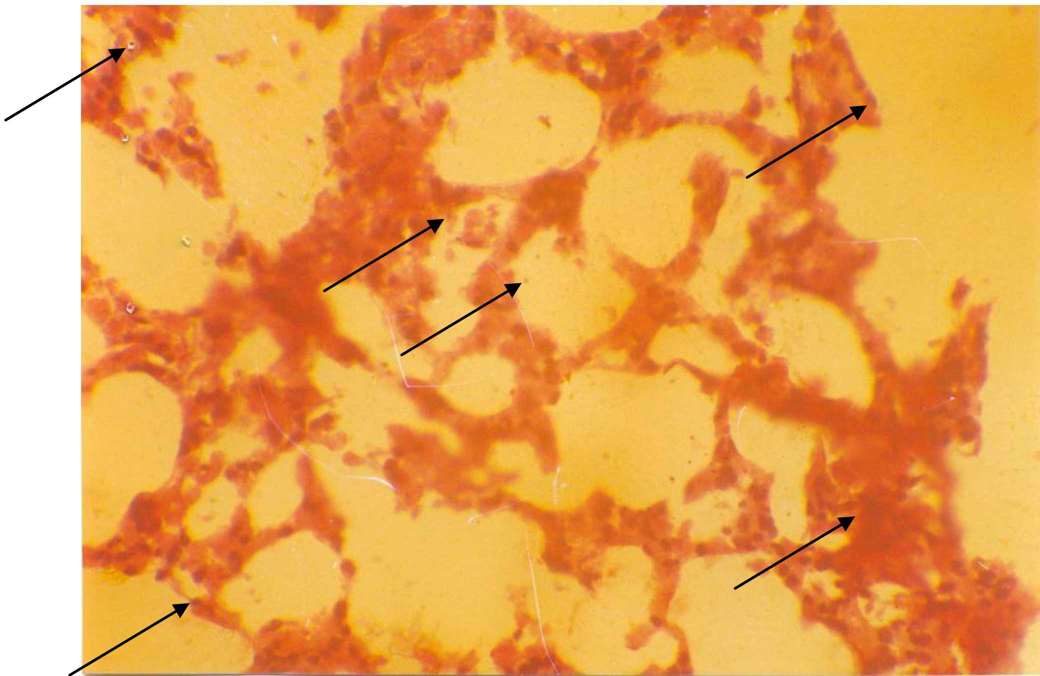


Plate 16: Slide micrograph of the lung tissue of the infected mouse stained with H & E showing short rods (*L. monocytogenes*), haemorrhages and mild oedema with lyphocytic infiltration within the bronchial wall which is fibroblastic

4.4.3 Re-Isolation *L. monocytogenes* 4b form the Experimental Infected Albino Mice

The test organism *L. monoctogenes* 4b was recovered from liver and lung that were homogenized and cultured in pre-enrichment broth, primary *Listeria* selective enrichment broths and selective solid media (Oxford, Palcam and Nalidixic acid Sheep Blood Agar).

4.5 ANTIBIOTIC SUSCEPTIBILITY

The *L. monocytogenes* 4b isolate was sensitive *in-vitro* to Gentamycin, followed by Ampicillin and then Penicillin. Others were Enrofloxacin followed by Neomycin, Oxytetracycline and then Keproceryl. However, the organism was resistant to Pefloxacin respectively. The sensitivity (antibiogram) pattern is shown on Plate 17.

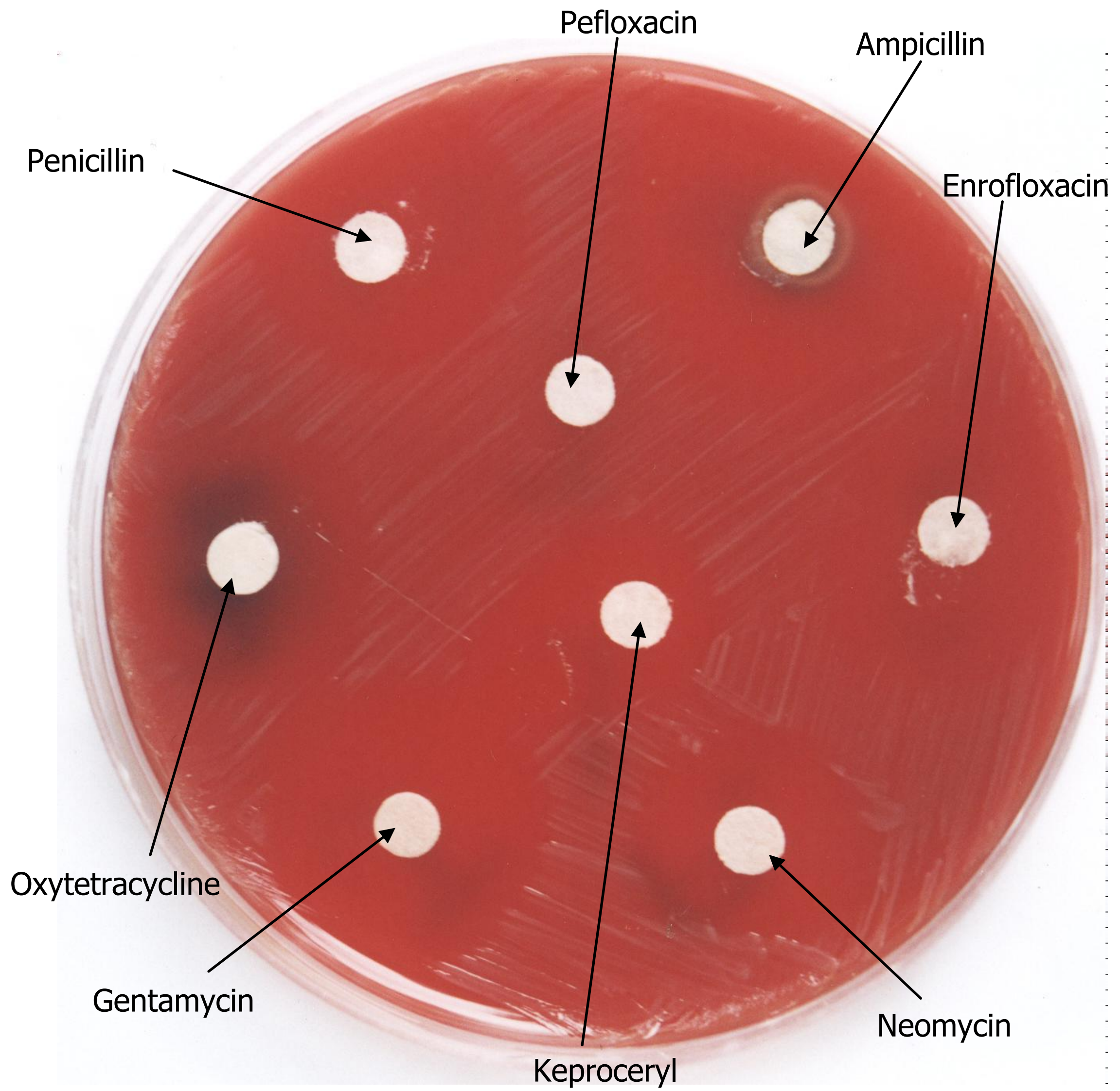


Plate 17: The Antibiotic Susceptibility of *L. monocytogenes* against each antibiotic on a disc showing Potency by inhibition zones

Generally, the 189 *Listeria* isolates out of the 900 samples comprising cow dung, goat faces, sheep faeces, rabbit faeces, poultry droppings, soil, green vegetation, human faeces, farm debris and water analysed using ANOVA and Chi Square (X^2) Test was highly significant at 5% and 10% level of probability (P = 0.01; P = 0.05) (Table 17). On speciation, the statistical analyses using ANOVA and Chi Square (X^2) Test showed that *Listeria monocytogenes* isolates among the 900 experimental samples investigated were significantly high as against other *Listeria* species at level 5% and 10% of probability (P = 0.01; P = 0.05) with the exception of human faeces and soil samples (Table 17). This was followed by *Listeria ivanovii* which was also significant in cow dung, sheep faeces, rabbit faeces and poultry droppings at level 5% and 10% of probability (P = 0.01; P = 0.05) (Table 17). The other *Listeria* isolates *L. seeligeri, L. welshimeri, L. grayi, L. innocua* and *L. murrayi* from the 90 different experimental samples each of cow dung, goat faces, sheep faeces, rabbit faeces, poultry droppings, soil, green vegetation, human faeces, farm debris and water were also significant at 5% and 10% level of probability (P = 0.01; P = 0.05), while some were not significant at the same level of probability (Table 17).

Table 17: The Chi Square (X^2) Test for Different Organisms Found in the Various Samples Investigated

Organisms \ Samples	Cow Dung	Farm Debris	Goat Faeces	Green Vegetation	Human Faeces	Poultry Droppings	Rabbit Faeces	Sheep Faeces	Soil	Water
L. grayi	NS 9.6	** 37.44	NS 0.237	* 13.23	NS 9.68	NS 3.947	NS 4.80	NS 0.1	NS 4.96	NS 1.14
L. innocua	NS 0.289	NS 1.052	NS 0.267	NS 2.56	NS 0.144	NS 0.26	** 48.13	NS 1.13	NS 8.90	NS 3.94
L. ivanovii	** 20.45	NS 5.037	** 17.91	NS 2.59	NS 5.897	** 23.655	* 17.68	** 33.48	NS 4.62	NS 0.49
L. monocytogenes	** 39.43	* 14.743	** 29.13	** 23.212	NS 11.328	** 18.97	** 27.14	** 54.17	NS 8.79	* 15.1
L. murrayi	NS 2.34	NS 2.152	MS 3.639	NS 3.31	NS 6.312	NS 2.766	NS 1.1	NS 1.58	** 83.24	NS 4.96
L. seeligeri	NS 0.133	** 24.711	NS 0.1	NS 7.45	* 13.058	NS 6.453	NS 0.2	NS 0.17	*** 101.213	NS 0.08
L. welshimeri	NS 1.17	** 23.98	NS 0.672	NS 1.81	NS 0.177	NS 8.82	** 22.6	NS 5.45	NS 0.95	** 37.07
TOTAL	*** 73.41	*** 71.67	*** 51.96	*** 54.16	*** 46.59	*** 64.87	*** 121.69	*** 96.05	*** 212.67	*** 62.76

KEY:
NS - Not Significant
* - Significant at 5% level of probability (P = 0.05)
** - Significant at 5% and10% level of probability (P = 0.05 and P = 0.01)
*** - Highly significant at 5% and 10% level of probability (P = 0.05 and P = 0.01)

Among the different solid media (Oxford *Listeria* Agar, PALCAM, and Nalidixic Acid Sheep Agar) used for the isolation showed that the isolation rate between Oxford *Listeria* Agar and PALCAM were not significant at P = 0.05 using the Duncan Multiple Range Test. Although there was no significant difference between Oxford Listeria Agar and PALCAM at P = 0.05 and p =0.01. However, when Oxford Listeria Agard and PALCAM are compared with Nalidixic Acid Sheep Agar it was significant at P = 0.05 (Table 13). The details of the statistical analyses are shown in Appendix B.

CHAPTER FIVE

DISCUSSION

The results of this study have shown the presence of *Listeria* species in the experimental specimens. The statistical analyses carried have shown that the occurrence of these bacteria on the experimental samples was significant at 5% and 10% level of probability (P = 0.05; P = 0.01). *L. monocytogenes* was found to have the highest percentage frequency of occurrence (8.6%). It was followed by *L. ivanovii* (4.2), then by *L. grayi* (3. 0%), then by *L.welshimeri* (1.8%), then by *L. innocua* (1.4%), then by *L. murrayi* 11(1.2%) and finally by *L. seeligeri* (0.7%). This is in agreement with the findings of (Arvanitidou *et al*., 1997 and Fenlon, 1999).These workers reported that *L. monocytogenes* and other *Listeria* species are widely present in the environment.

These authors and others also reported that *L. monocytogenes,* other *Listeria* species ands other pathogenic microorganisms have frequently been isolated from animal food stuffs, human, livestock (cow, goats, sheep and rabbit) faeces, soil, manure compost and water sources (Welshimer, 1968; Waltkin and Sheath, 1981; Weis and Seeliger, 1975; Schlech *et al.,* 1983; Fenlon, 1985; Farber and Peterkin, 1991; Schuchat *et al*., 1991;Pinner *et al*., 1992; Dyer and Stoltenow, 2002; Bailey *et al*., 2003 and Islam *et al*., 2004).

The results have confirmed that green vegetation, water sources, the soil and human and domestic animals faeces are potential sources of *Listeria monocytogenes* and other *Listeria* species. These are thus, potential sources of human infection in Nigeria. Normal free movement of the animals must have

aided in the spread of *Listeria monocytogenes* and other *Listeria* species isolated in the experimental specimens. This stems from the faecal contamination of the vegetation and soils of thel study areas. Similar observations were made by (Palmgren *et al*., 1997; Osterlund and Tauni, 2000). They reported that free movement of birds and their associated wastes were potential sources human and animal pathogens.

5.1 ISOLATION OF *LISTERIA* SPECIES FROM DIFFERENT EXPERIMENTAL SAMPLES

Rabbit Faeces

The Rabbit faecal samples yielded the highest number of *Listeria* species isolates. The percentage occurrence of this pathogen in the faecal samples was 33.3%. The statistical analysis carried showed that the occurrence of these bacteria is highly significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The *Listeria* isolates included *L. monocytogenes* which was found to have 12.2% occurrence, *L.ivanovii* which had 6.7% occurrence, *L. innocua* which had 5.6% occurrence*; L. welshimeri* which had 4.4% occurrence; *L. grayi* which had 3.3% occurrence, *L. murrayi* which had 1.1% occurrence and *L. seeligeri* which had 0% occurrence. These findings are similar to the results obtained by (Murray *et al.,* 1929 and Gray and Killinger, 1966). These workers were the first to report that *L. monocytogenes* and other *Listeria* species as a pathogen in Rabbits.These authers, thus reported that *L. monocytogenes* and other *Listeria* species are frequently found in Rabbits. The implication of the findings in this study is that Rabbit might be major sources of contamination and spread of *Listeria* species.

Sheep Faeces

The Sheep faecal experimental specimen yielded second isolation rate of *Listeria* species, of a percentage occurrence of 27.8%. The statistical analysis carried out showed that the occurrence of these bacteria (27.8%) is significant at 5% and 10% level of probability (P = 0.05; P = 0.01). On speciation of the *Listeria* isolates, the percentage occurrence showed that *L. monocytogenes* had 14.4% occurrence; followed by *L.ivanovii*, which had 7.8%; then by *L. welshimeri*, which had 2.2% then *L. innocua*, which had 1.1%. The occurrence of other *Listeria* species were *L. grayi*, 1.1% *L. murrayi*, 1.1% which had occurrence respectively and *L. seeligeri* which had 0% occurrence. This is in agreement with the reports of (Gill, 1933; Gray and Killinger, 1966; Schlech *et al* 1983; Wilesmith and Gitter, 1986 and Bailey *et al.,* 2003)) who reported the susceptibility of Sheep to *L. monocytogenes.* The authors reported the high carriage of *L. monocytogenes* and other and *Listeria* species in the intestine of sheep. This led to the contamination of cabbarges as a result of application of the contaminated sheep feaeces as manure on the cabbarge farm land. These results thus, implicate contaminated sheep faeces as plaussible vehicles for the spread of *Listeria* bacteria in the environment.

Soil

The soil experimental samples from farm land gave the third highest occurrence of *Listeria* species out of 90 soil experimental specimens.The percentage occurrence was 24.4%. The statistical analysis carried out have shown hat the occurrence of these bacteria is significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The percentage occurrence distribution of the

isolates revealed that *L. monocytogenes*, had 6.7% occurrence, then followed by *L. murrayi*, 5.6% and then *L. seeligeri* 4.4%. Others were *L.ivanovii*, which had 3.3%, then *L. innocua* which had 2.2%); and *L. grayi* and *L. welshimeri which had* 1.1% each respectively.The health implication of this result is that the experimental soil specimens are not void of *Listeria* species.This may be as a result of previous application animal faeces on the land or from free range wild and domesticated livestock and poultry.Therefore, the farm land humus soil by this study is a major source of *Listeria* species contamination of food or water in our locality.

This is in agreement with the findings of Weis and Seeliger (1975) Fenlon (1985) that *L. monocytogenes* and other *Listeria* species to be widely spread in farm land soil. Also, several studies have established the relationship between livestock movement and the development of several diseases with common epidemiological features and geographical distribution.

The organisms inhabit the soil especially the humus which could contain faeces of various animals (Schlech *et al.*, 1983; Fenlon, 1985 and 1986). Farber and Peterkin, (1991) reported that *L. monocytogenes* can be tranmitted through soil and vegetable sources. The inference of this finding in this study is that crops, vegetables or creeping plants could easily be contaminated with *Listeria* organisms from the sub, top soil and grasses when in close contact in the farm.

Farm Debris

The farm debris experimental specimen investigated yielded significant *L. Listeria* species.The percentage occurrence was 23.3% out of the farm debris

experimental specimens examined. The statistical analysis carried out showed the occurrence of these bacteria to be significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The distribution occurrence of the Listerial isolations revealed that *L. monocytogenes* had 7.8%. *L. monocytogenes* was then followed by *L. grayi* which had 4.4% and then *L innocua* and *L. ivanovii* which had 3.3% respectively. Other ocurrences were *L. seeligeri* which had 2.2%, followed by *L. murrayi* and *L. welshimeri* which had 1.1% each. These results are in agreement with the reports of Welshimer (1968)); Blenden *et al.,* (1987) Schuchat *et al.,* (1991) and Fenlon (1999), who cultured *L. monocytogenes* and other *Listeria* species from farm debris.

The significant health implication of the results in this study will explain three things: the application of untreated animal faeces contaminated with *Listeria* species on agricultural farm lands as manure purposely by individuals and indiscrenate defaecation on the farm lands animals and humans. Also the heat resistance of the bacteria.This means that untreated animal faeces imposses environmental health hazards.

Cow Dung

The results of the presence of *Listeria* species on the experimental cow dung was significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The distribution occurrence percentage rate was 22.2% out of the experimental specimens.The breakdown of the distribution percentage showed that *L. monocytogenes* isolates were 11.1%.This was followed *by L.ivanovii* which had5.6%, then by *L. grayi* which had 3.3%.The percentage occurrence of other

Listeria species were *L. welshmeri* which had 1.1%, *L.murrayi* 1.1%, *L.seeligeri* 0% and *L. innocua* 0%. These results are in agreement with the findings of Blenden *et al.,* (1987), Fenlon (1999) and Bailey, *et al.,* (2003) who reported significant *L. monocytogenes* and other *Listeria* species cultures from cow dung. The findings in this study have revealed contaminated cow dung as health hazards in the environment and to susceptible individuals.

Poultry Droppings

The result of the investigation of the occurrence of *L. monocytogenes* and other *Listeria* species in the poultry droppings revealed that the bacteria were significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The occurrence rate was 18 (20.0%) out of 90 experimental specimens. The percentage distribution indicated that *L. monocytogenes* isolates were 7.8% followed by *L. ivanovii* which had 5.6% isolates.The other percentage isolates were *L. grayi* which had 2.2% then followed by *L. weshimeri* which also had 2.2% respectively.Others were *L. seeligeri* which had 1.1%, *L. murrayi* which had1.1% and *L.innocua* which had 0%.. This result is in agreement with the reports of Ali and Ibrahim (1992) who reported harbouring of *L. monocytogenes* and other *Listeria* species in the gastrointestinal tract of most chickens examined without overt listeriosis.

Palmgren *et al.,* (1997), Fenlon (1999) and Duarte *et al.*, (2002), also, reported faecal carriage of *Listeria* species by seagulls and wild birds feeding on sewage sites. The health implication of the practice of the application of domesticated faeces as bioorganic fertilizer is very harmful.This because, without

treatment as being practised in the present day Nigeria by most vegetable farmers is a means of contaminating the agricultural land. This no doubt will contaminate vegetables that are in most cases not cooked befoer consumption.

Green Vegetation

The isolation of *Listeria* species from the experimental green vegetation specimens was significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The percentage rate of occurrence was 17.8%. The speciation occurrence showed that *L. monocytogenes* had 7.8%, followed by *L. grayi* which had 3.3% and then by *L. ivanovii* which had 2.2%.The percentage occurrence of other *Listeria* isolates were *L. innocua* which had 1.1%, *L. murrayi* which also had 1.1%, then *L. welshimeri* which had 1.1% and *L. seeligeri* 1.1% respectively. The results obtained in this green vegetation study are in agreement with the findings of (Welshimer, 1968; Waltkin and Sheath, 1981; Weis and Seeliger, 1975; Fenlon, 1985).

These authors reported the isolation *L. monocytogenes* and other *Listeria* species from vegetion, soil and agiricultural land. The investigation has revealed that the vegetation of the studied areas harbour *Listeria* species. This means that humans and domesticated animals can easily contract the bacteria via the green vegetation. Also, the inference of the finding in this study is that grasses, crops, vegetables or creeping plants could easily be contaminated with *Listeria* bacteria from the soil when in close contact.

Goat Faeces

The goats' faecal experimental specimens yielded 16.7% occurrence rate of *Listeria* species. The statistical analysis carried out showed that the occurrence is significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The occurrence of the different *Listeria* isolates showed that *L. monocytogenes* had 8.9%, followed by *L.ivanovii*, which had 4.4 and then by *L. grayi* which had 2.2%. Other *Listeria* species occure as follows; *L. welshimeri* 1.1%, *L. innocua* 0%, *L. murrayi* 0%, and *L. seeligeri* 0%.This is in agreement with the reports of (Gill, 1933; Gray and Killinger, 1966; Schlech *et al* 1983; Wilesmith and Gitter, 1986 and Bailey, *et al.,* 2003)) who reported the isolation of *L. monocytogenes* and other *Listeria* species. The authors reported the carriage of *L. monocytogenes* and other and *Listeria* species in the intestine of goats. This led to the contamination of cabbarges as a result of application of the contaminated goats' feaeces as manure on the cabbarge farm land. These results thus, implicate contaminated goats'faeces as plaussible vehicles for the spread of *Listeria* bacteria in the environment.

Pond Water

The percentage rate of occurrence of *L. monocytogenes* and other *Listeria* species in the pond water was 13.3%. The result of the isolation is significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The distribution pattern of the *Listeria* species isolates revealed that *L. monocytogenes* had 5.5% occurrence, while *L. welshimeri* had 3.3% and *L.ivanovii had* 1.1%.The species, *L. grayi* had 1.1%, *L. innocua* 1.1%, *L. murrayi* 1.1%) and *L. seeligeri* 0%.This result is in agreement with the findings of Geuenich *et al*., (1985) and Müller

(1990), who also reported the occurrence and isolation of *listeria* species in waste water.

However, excessive distribution of the animal wastes in the environment and runoff water from contaminated agricultural land and vegetation must have contributed to the release of the *Listeria* bacteria into the pond waters. The health implication of these ponds is that the ponds which serve as source of domestic and drinking water might be major source of infections to humans and the contamination of edible vegetables.

Human Faeces

The percentage occurrence of *L. monocytogenes* and other *Listeria* species in human faeces was 11.1%.from experimental specimens. The statistical analysis showed that the occurrence is significant at 5% and 10% level of probability (P = 0.05; P = 0.01). The distribution pattern indicated that *L. monocytogenes* isolates were 4.4% follwed by *L. ivanovii* which had 2.2% and then by *L. grayi* which also, had 2.2%. The other isolates were *L. seeligeri* 1.1%, *L. murrayi* 1.1%, *L. innocua* 0% and *L. weshimeri* 0%.The results are in agreement with the findings of (Kampelmacher and Van-Noorle Jansen, 1969; Bojsen-Moller, 1972) who reported *Listeria* species in faeces of slaughter house workers.

Also, Geuenich *et al*., (1985); Schuchat *et al*., (1992); Pinner *et al*., (1992) reported the occurrence and isolation of *listeria* species from faeces of patients with diarrhoea and healthy food handlers. Although the health status of the individuals whose experimental faecal samples were examined, were not known,

the poor personal hygiene and eating habits of the persons must have contributed to their harbouring thebacteria.This has revealed that there are assymtomatic carries of the *Listeria* bactera in the study population. This means that proper diposal of human is very imperative to avoid epidemics as these wastes are liable to the contamination of the environments with *Listeria* bacteria or leachates through the soil.

5.2 METHODS FOR THE DIFFERENTIATION OF *LISTERIA* SPECIES

The ecological niche of *L. monocytogenes* and other *Listeria* species in the environment is difficult to define. The applied microbiology approach to studies of microorganisms of interest (like in this study) is much the same way as the clinical microbiology approach in culturing and studying of human pathogens. However, when specific pathogens are to be isolated from clinical, environmental and food sources competitive microflora or other organisms not wanted are purposely suppressed and the desired micro organism(s) is/are selected or isolated in pure culture for studies.

It was observed that the temperatures, PH, moisture contents and dry matters of experimental specimens influenced the isolation of *L. monocytogenes*, the other *Listeria* species and other microorganisms.This is in agreement with the findings of Fenlon, (1985,1986);Blenden *et al* ., (1987); Juntilla *et al.,* (1988) who reported the influence of these exogenous factors for the growth of Listeria bacteria.

In this study, the optimization of the classical methods in the laboratory adopted enhanced the isolation of *L. monocytogenes* and the other *Listeria*

species from the natural ecological experimental spcimens. The isolation of *Listeria* organisms entailed the use of selective and differentiative media. The former being media in which the organisms can grow in preference to other organisms. The latter media separate the organism by the reactions defining certain biochemical characteristics.

5.3 SOLID MEDIA FOR THE ISOLATION OF *LISTERIA* SPECIES

Generally, *L. monocytogenes* and other *Listeria* species were best detected on Oxford and Palcam followed by blood agar medium. The colonial study of *Listeria* species on the agar plates and microscopy were excellent for their tentative identification, even in the presence of large number of other organisms. Nevertheless, some colonies that closely resembled *Listeria* species were never eliminated on the basis of their Gram's stain reactions, motility and other biochemical reactions.

On moulds contamination, not many problems were encountered. This however, could be as a result of polymxyin B and cycloheximide content of the broth media and agar plates. Moreso, the pH of the broth media did not vary much during the cold enrichment/incubation (pH 7.8 – 8.2), for 7 days (168hours).

Among all the solid media employed for isolation, the sensitivity for the isolation of the experimental bacteria, *Listeria* monocytogenes and other *Listeria* species were Oxford *Listeria* agar, Palcam agar and Nalidixic Sheep blood agar plates. The most effective of the media was the Oxford *Listeria* agar followed by Palcam agar and then by Nalidixic Sheep blood agar.

The isolation efficiency between Oxford *Listeria* agar and Nalidixic Sheep blood agar plates was significant at 5% and 10% level of probability (P = 0.01; P = 0.05). Also efficiency between Palcam agar and Nalidixic Sheep blood agar plates was at a probability of P = 0.05 and P = 0.01.

However, on the rate of *Listeria* species from the experimental specimens there was no significant difference between the Oxford *Listeria* agar and Palcam agar plates at a probability of P = 0.05 and p =0.01.These results have revealed that the none isolation of *L. monocytogenes* and other *Listeria* species from clinical, food or environmental samples in most laboratories is due to lack of appropriate techniques and correct culture media.

The significance efficiency of Oxford *Listeira* and Palcam agar over the Nalidixic Sheep blood agar plates is in agreement with the findings of Art and Andre (1991). They reported that Oxford *Listeria* and Palcam agar were better than blood agar with nalidixic acid for the detection of *L. monocytogenes* and other *Listeria* species from samples. The utilization of selective inhibitory constituents like lithium chloride, acriflavin, colistin sulphate, cefotetan, cycloheximide and fosfomycin and the indicators aesculin and ferrous iron in Oxford *Listeira* and Palcam agar must have influeced n the rate of isolation.

On Oxford *Listeria* agar, *Listeria* species hydrolyse aesculin to aesculitin producing black zones around the colonies due to the formation of black iron phenolic compounds derived from glucon in the medium. Typical *Listeria* colonies are almost always visible after 24hr; nevertheless s, incubation was continued for 48hrs to detect slow growing strains. In contrast, on the Palcam agar, *Listeria* colonies appeared grey-green and black-sunken centres. Also a black – halo

against a cherry red background colour of the Palcam medium while on Nalidixic Sheep blood agar plates Listeria species exhibit beta- haemolysis or alpha haemolysis.

On the course of the laboratory investigations, some minor variations were encountered in the results of tests used in confirming and differentiating *Listeria* bacteria. Such variables were bacilli, long rods, Chinese-like letters and coccoid forms on Gram's stained reactions. These atypical behaviours were later observed to be as a result of depletion nutrients in the media utilized, the pH, growth temperature and age of the culture. These findings are similar to the reports of (Gray and Killinger, 1966; Van-Netten *et al*., 1989; Curtis *et al*., 1989).The authors also, reported tese variables aid the bacteria to survive in an adverse environment.

It was observed in this study, that Gram's reaction of *Listeria* is sometimes similar with *Diphtheroides*, *S. aureus* and *Corynebacteium* species. Also some cultures of *L. monocytogenes* on blood agar showed a cream whitish colouration which is typical of *Diphtheroides* and *S. aureus*. These observations are needful for proper laboratory differential diagnosis as growth on blood agar and Gram's reactions may not give an accurate character of the organism.

Moreso, this has shown that a good number of cultures in the laboratories must have been discarded as *Diphtheroides* of no significance, or at best treated as *S*. *aureus* as both *Listeria* species and *S. aureus* are both catalase and coagulase- positive. Therefore, it is imperative that appearance on blood agar is a feature not related to *Listeria* species on clinical morphology (macroscopic).

The isolation of other bacteria and fungi in this study, using *Listeria species* selective broth agar plates reveals that they can also support their growth. However, this could be as a result gene competence or intergeneric relationship between *Listeria* species and the other microorganisms in the ecological nitche. These genes can be transferred through conjugation (or the mating process), transduction (transfer with the help of viruses) and transformation (direct uptake of DNA by the microoganisms within the ecological system in the experimental specimens.

5.4 DIFFERENTIATION OF*LISTERIA MONOCYTOGENES* INTO SEROTYPES (SEROVARS) USING POLYMERASE CHAIN REACTION

Generally, Gram-positive bacteria are more resistant to lysis than Gram-negative bacteria, because of the nature of the cell wall. However, this problem was successfully circumvented with *Listeria* bacteria by the use of lyzozyme and proteinase k after treatment with acetone. In this study, this method was used successfully for the lysis of *Lsteria* cells.The procedure was very effective for obtaining sufficient amounts of proteins from the bacteria.

Agar gel electrophoresis of the whole-cell proteins produced different migration patterns depending on their molecular weight. The use PCR for the classification of some of the *Listeria* bacteria into *L. monocytogenes* and other *Listeria* species isolates in this study gave a clearcut differentiation. The PCR also classified *L. monocytogenes* and other *Listeria* isolated in this study into serotypes. This shows that the application of *Listeria* proteins' profiles of the bacteria isolated in this study can be used for the idrentification of *Listeria* species. These results are in agreement with the findings of Rocourt *et al.*, (992);

Bubert *et al.*, (Bubert *et al.,* 1992;1994) that Listeria species can be separated more distinctly by genetic methods.

In conclusion, the use of PCR methods for the molecular characterization of *L. monocytogenes* and other *Listeria* isolates in this study has been fundamental to the understanding of the distribution of pathogenic *L. monocytogenes* serotypes or serovars that are common in Nigeria especially in the study area. Some of these serotypes of *L. monocytogenes* (4b) are virulent. If these serovars are exploited, they may be useful for vaccine development against listeriosis.

5.5 PATHOGENICITY (INFECTIVITY) STUDY

In bacterial pathogens, disease process or spread in individuals is often insidious or regular, that is a definite cause of expression in organs and subsequently clinical manifestations is usually observed. This is sometimes not so in listeriosis as different hosts respond differently on infection.

The immunocompromised mice infected with Nigerian field isolates of *L. monocytogenes* serotype 4b all died. The positive control mice also died all within 3-5 days potinfection.The negative control mice survived. The results of this experiment have revealed the virulence of Nigerian field isolates of *L. monocytogenes* serotype 4b. The experimental albino mice came down with diarrhoea and septicaemia leading to death due to infection by the experimental *L. monocytogenes*. The results of this study are in agreement with the reports of Stelma *et al.,* (1987) and Mielke *et al.*, (1993), who reported the resistance of *Listeria monocytogenes* against human or animal neutrophiles. They reported that the inability of the neutrophiles to kill the *Listeria* does not relate to poor

ingestion of the organism by the macrophage, but neutralizing properties of *Listeria monocytogenes* to all defense mechanisms of the macrophages with the aid of listeriolysnon O.

The listeriolysin (LLO) is the virulence factor of *L. monocytogenes* that elicits the T – cell response is heat liable protein, and often destroyed when cells are heated. This is similar to the test organisms as the immunologic systems of the experimental mice has been compromised allowing listeriolysin O, haemolysin and invasive associated protein (*iap*)-gene (p60 protein) produced by the *L. monocytogenes* to in the neutralization of immune defense system of the infected albino mice host used.

In a different investigation, Hof, (1991) reported that *L. monocytogenes* has been resistant to superoxide radicals and hydrogen peroxide which are defense mechanisms of the macrophages. This further supports the findings of this study as the test organisms were catalase positive *in vitro.* This reaction must have occurred *in vivo* in the experimental mice producing superoxide dismutase and hydrogen peroxidase enzymes that aided the organisms to circumvent the mice *in vivo* defense mechanisms.

As observed in this study, it is adjudged that these *Listeria monocytogenes* tested had virulent and invasive factors that caused the clinical signs and death of the infected mice. This no doubt will be applicable to human infection among the Nigerian population, who are immunocompromised. Although not considered in this experiment, other factors like pH, osmolarity and concentration of certain metallic ions (Sword, 1966; Tilney and Tilney, 1993; Puttmann *et al.*, 1993),

which influence the virulence of *Listeria monocytogenes* in susceptible hosts must have contributed to virulence of the experimental *L. monocytogenes*.

The recovery of *Listeria monocytogenes* from the organs and its intracellular presence in the the lungs and liver tissues of the experimental mice is a proof that the animals died as a result of the infection. In conclusion, from this study, there is evidence that Nigerian field isolates of *Listeria monocytogenes* 4b express virulent characteristics that aid the organisms to circumvent the host immune system causing murine listeriosis. The murine listeriosis as observed in this case, is applicable to individuals with weakened immune systems (AIDS, cancer patients, pregnant Women, the young and the elderly) in our population when infected with virulent *L. monocytogenes,* as the organisms elicit host reactions which are very similar in man and rodents.

The experimental *Listeria monocytogenes* isolated in this study neutralized the defense immunity mechanisms in weakened the albino mice system leading to cause their infection and death. The resistance to *L. monocytogenes* infection is independent humoral defence mechanisms as passive transfer of immune serum fails to protect the host against *Listeria* infection. Sheehan *et al.,* (1994) reported the recovery from a primary *Listeria* infection due to resistance to interferon, and protective immunity mediated by anti-*Listeria*-specific T-cells. The activation, clonal expansion and mobilization of anti-*Listeria* specific T-cells (CD8 and CD4) has been found to be as a result of the ability of *L. monocytogenes* to survive and replicate in resident macrophages (Kaufmann, 1993).

Also, the results of this investigation are similar to that of Mielke *et al.,* (1988).The authors reported that non-specific defence mechanisms play a major role in suppressing the multiplication of *L. monocytogenes* after host's cells invasion. Thus, the organism is often killed in rat serum, but not in the mouse, mice or human serum.The age of the mice contributed to being infected. Mielke and colleagues also reported that low gastric acidity in babies and old has been reported to facilitate the passage of viable *Listeria* into the small intestine.

The results of the liver and lung tissues of infected experimental albino mice with *L. monocytogenes* 4b revealed prominient lysis of paramchymal cells. This is because in figthting invading organisms, the cellular components on resistance and immunity: the polymorphonuclear leukocytes represent the first line of defence as they are often found very early on an infectious focus.This in agreement with findings of Njoku-Obi and Osebold, (1962); Heymer *et al.,* (1988). The authors reported that the bacteria are responsible for the lysis of paramchymal cells infected with *L. monocytogenes*. Therefore, to liberate and subsequently phagocytose the organism by potent effector cells such as macrophages, the elimination of these phagocytes definitely impairs the defence. Some of these events that occur in murine hosts following infection with *L. monocytogenes* are likely to be the same as in human infections.

In this study, some exogenous factors such as pregnancy and age enhanced the susceptibility of the experimental albino mice as the pregnant ones came down with listeriosis faster than the nonpregnant mice. This is similar to the findings of Wirsing von Konig, *et al*., (1988).They reported that it could be

influenced by the organism itself and a wide array of endogenous and exogenous host factors. Using experimental infection of murine hosts, they reported that the most important in inbred mice strains showed susceptibility or resistance to the organisms in relation to the age which was crucial for the outcome of the infection. Aged mice were found to possess an increased non-specific resistance as compared to the young adult animals. They also reported that susceptibility is influenced by the nutritional status or by a simultaneous second antigenic stimulation.

On exogenous factors, the authors reported that macrophage blocking agents could totally abolish resistance to listeriosis. However, the non-specific defence mechanisms are normally effective in controlling infection and a lot of immunomodulating agents like BCG, killed *Bordetella petusis*, *Propionbacterium acnes* organisms, lipopolysaccharides and suramin can either decrease or increase the resistance to listeriosis.

Nevertheless, what makes the old, young, pregnant and immunenocompromised individuals or hosts more susceptible to this opportunistic organism to take advantage, is the dampening effect that the immunosuppressive agents have on the T-cell system.

5.6 ANTIBIOTIC SUSCEPTIBILITY

The organism was sensitive to a good number of antibiotics *in vitro*. The sensitive regimen was Gentamycin as most sensitive, followed by Ampicillin and then Penicillin. Others were Enrofloxacin followed by Neomycin and then Keproceryl. However, the organism was mildly sensitive to Oxytetracycline and

resistant to Pefloxacin respectively. The degree of sensitivity of *L. monocytogenes* to any drugs was judged by comparison of zones of inhibition among the antibiotics. The implication of these findings is that there a wide range of antibiotics available to administer wthen listeriosis diagnosed early in our locality.

Generally, the results of this study have demonstrated that pathogenic *L. monocytogenes* and other *Listeria* species are commonly carried by livestock, poultry, water and humans. The pathogens were diseminated in agricultural lands. Therefore, this information is vital to the farmers, the agriculturist, Veterinary and medical communities. This also, calls for concerted efforts and common approach to prevent the spread of fatal foodborne pathogens.

However, there is no doubt that the persistence of poverty especially in developing countries like ours along with overpopulation, environmental degradation and poor agricultural policies, must have contributed to the persistence of the organisms in the study areas- environment and farm produce. Until this is checked, the incidence of certain food-borne organisms like *Listeria* will continue to increase because of changing agricultural, social and economic systems. Even large scale or epidemic mortality resulting from listeriosis in human and animal populations sometimes goes unnoticed in most parts of Nigeria. This is probably due to of lack of effective and active surveillance and diagnostic efforts.

5.7 SUGGESTED CONTROL AND PREVENTIVE MEASURES ON THE SPREAD OF *L. MONOCYTOGENES* AND OTHER *LISTERIA* SPECIES

The sole aim of the microbiological monitoring of food, animals and their products, plants or vegetables and water is to protect the health of the community and individuals by preventing the spread of infectious and non-infectious diseases and their causative agents through environmental sources.

The biggest threat in the control of *L. monocytogenes* is in the animals, their products, vegetables and related products, often eaten without adequate heating as animals can range from totally normal carriers, wherein they demonstrate no clinical signs oflisteriosis. These animals often shed the organism into the environment, hence contamination of food, water or other animals in the farm.

However, the control of *L. monocytogenes* other *Listeria* species in the environment; agricultural land, food or processing industry is often very tedious. This is because of the intracellular (obligate), complexity of the organisms' life histories. They can survive in adverse environments sources as revealed in this study. In a herd, there is also an ample chance to cross-infect during breeding process. The commercial channels such as abattoirs, retail or wholesale outlets of the contaminated animals, animal products and vegetables can also serve as means of Listerial spread. Therefore, any place, farm and food industry that has been contaminated should thoroughly be sanitized to prevent biofilms formation by the organisms.

In the case of dairy products, the source of the post production contamination by *L. monocytogenes* is often from the processing environment. Therefore, environmental contamination by *L. monocytogenes* through faeces of animals and man would be possible sources of contamination of fresh market produce (vegetables). Since the organism has the propensity for survival under adverse conditions, to mitigate the potential for the survival and building up of *L. monocytogenes*, attention needs to be focused on the natural sources of contamination by treatment of the animals in the farm and adequate pasturisation of dairy products.

Furthermore, research is needed on several aspects of the epidemiology *L. monocytogenes* using polymerase chain reactions directly on sample materials. The use of this more reliable, sensitive and standard technologies are very imperative. This is because, although it is important to verify safety through food inspection where possible, an ability to identify the sources of environmental contamination is critical to circumvent reoccurrences. These improved new diagnostic tools used on this study are essentially needed for regular epidemiological surveillance of people, livestock, poultry and the peculiar ecological sites of the environment. This will enhance the early identification of possible contamination sources and adequate measures taken.

Effective solutions of these problems is the elimination of the threats at the production (or pre harvest) stages (farm and storage). In essence, these contaminations are amenable to control by improved management of animal and human wastes, sanitation and food production in Nigeria.

5.8 CONCLUSION

Based on the findings, this study has amassed evidence that enabled me to reach the conclusion that livestock and humans in Nigeria are carriers of *Listeria monocytogenes* and *Listeria* species. Therefore, the use of livestock wastes in agricultural environment like crop production without adequate treatment could be very hazardous. It is a well known fact that the agricultural policies in Nigeria basically focus on the promulgation and promotion of food security for the increasing population, family incomes and enhancement of general wellbeing of the citizenry. However, little regard is paid to what practices are adopted and the nature of their effects on the environment and fresh farm produce.

Therefore, knowledge of the distribution of the *Listeria* organisms in the agricultural environment is vital to the evaluation of possible *Listeria* contamination of farm produce. This knowledge about the presence of Listeria in the ecological peculiarities acquired in this study, will no doubt aid in clinical epidemiologic investigation as possible sourcesof contamination in the time of outbreak of listeriosis in the populace. Also, the use of modern techniques on the isolation and identification of *Listeria* as in this study, will help our laboratories in the rapid detection and diagnosis of the disease.

It also was observed that *S. aureus* often share the same ecosystem with the *Listeria* species. Furthermore, on the investigation of the samples and media utilized, it was noted that other bacteria and fungi like *Brochothrix* species, *Kurthia* species, *Streptococcus* species, *Bacillus* species, *Mucor* species, *Candida albicans, Candida* species, *Penicillium* species and *Aspergillus* species can grow on

the *Listeria* selective media used in this study. It is therefore, concluded from this study that *Listeria* species co-habit the same ecological/ecosystem with other organisms.

In conclusion, the components of the environment are the atmosphere, the land, water, humans and other animals. All this interrelate in a balanced natural form or pattern. However, these interrelationships are mostly disturbed by human actions and activities. These actions, although are for development often bring about disadvantages to human health. While it is estimated that 90% of *L. monocytogenes* infection to man and animals are through food, there is still much to learn about how the environmental factors operate. Thus, health education on the effect of soil, possible farm produce and water pollution by *L. monocytogenes* need to be advocated in Nigeria. This will ensure cooperation from people to who all benefits of any research and control measures must be directed to.

5.9 SUMMARY OF FINDINGS

1. The findinings obtained through culture and also from the molecular characterization of *Listeria* isolates, have established one fact: That *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c, 3a, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Also *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi* and *L. grayi* sub-species *murrayi* exist in our environment. The livestock faeces, human faeces, farm debris, vegetation and water are unique environmental habitats for *L. monocytogenes* and other *Listeria* species in the study areas.

2. The production of extracellular invasive associated protein (p^{60}) which is a major virulent and pathogenic factors by *L. monocytogenes* is present in Nigeian field isolates.
3. The procedure using sugars and CAMP test (which are biochemical) for the differenciation of *L. monocytogenes* from other *Listeria* species is cumbersome and time- consuming compared to PCR. The PCR is simple and results can easily be interpreted. It is fast and eliminates fallacies between *L. monocytogenes* and related pathogens. This method is recommended for use in our laboratories for rapid diagnosis.
4. The pH, temperature and moisture content of the samples influenced the habitation of *L. monocytogenes* in them.
5. Ribbits and sheep are more carriers of *Listeria* than other animals sampled.
6. Pre-enrichment, selective enrichment broths and selective agar inoculation for the isolation of *Listeria* organisms from non-sterile samples are more effective than ordirinary media.
7. Co-habitation of *Listeria* organisms and other bacteria and fungi is common in the environment screened.
8. *Listeria monocytogenes* isolates were virulent to laboratory animals leading to death.
9. 9. The use selective solid media is efficient for the isolation of *Listeria monocytogenes* and other *Listeria* species.
10. 10 *Listeria monocytogenes* is sensitive to a number of commonly available antibiotics

5.10 CONTRIBUTION TO KNOWLEDGE

Results obtained from this work has immensely contributed to the epidemiology, phenotypic and genotypic characteristics and virulence of *L. monocytogenes* and other *Listeria* species in the study area in particular and Nigeria in general throught the following;

a Baseline information on the distribution and prevalence of *Listeria* organisms in some parts of Plateau State, Nigeria.

b Ecological niche of *Listeria* organisms in some parts of Plateau State.

c Molecular epidemiology of *Listeria organisms in Plateau State*.

d Optimization of isolation procedures of the *Listeria* organisms from biologically non-sterile samples.

e Revelation of specific *L. monocytogenes* serotypes (Serovars) and its significance in agriculture.

f The presence of invasive associated protein *iap* P^{60} in Nigerian field isolates of *Listeria monocytogenes*.

g Carrier Status of *Listeria* organisms, its implications in public health and livestock economies.

h Concurrent inhabitation of *Listeria* species and other microorganisms in the eco-microbiological system.

i The advantage of selective over non-selective *Listeria* media.

j Nigerian field isolates of *Listeria monocytogens* are virulent to susceptible hosts.

5.11 RECMMENDATIONS FOR THE PREVENTION AND CONTROL OF *LISTERIA* AND OTHER EMERGING PATHOGENIC BACTERIA

Surveillance is a key defense against emerging and re-emerging pathogens like *Listeria* species. Therefore, there is need for adequate provision of surveillance, diagnosis and disease reporting system with the collaboration of Medical, Veterinary, and Government Agencies such as (Wildlife, enviromnmetal, food manufactures/vendors and emergency services.This possible through the following.

Applied Research

Research is vital and essential to adequately understand emerging and re-emerging bacterial diseases and the aetiologic pathogens for the purpose of prevention and control. This requires provision of tools needed for identifying and understanding the pathogens. Applied research plans and priorities for the prevention and control of *Listeria* bacteria should include:

a) Strenghtening of basic and applied research on the pathogens, host and environmental factors that influence the survival and emergence.

b) Knowledge of the pathogen, host and environment interactions to enhance our ability to predict and prevent conditions that can lead to an sporadic outbreak or epidemic in a population of humans and animals.

c) Development of diagnostic techniques and procedures to detect and control *Listeria* bacteria and listeriosis.

d) Sequencing and post-genomics research of *Listeria monocytogenes* and animal and environmental vectors inorder to reveal the gene responsible for microbial/vector evolution adaptation and pathogenicity.

Collaboration

As *Listeria monocytogenes* 4b is an important foodborne pathogen of economic importance has emerged in Nigeria. The medical and veterinary communities' primary mission is to protect or treat humans, livestock and poultry industries from diseases that may threaten their existence. There is the need for co-ordinated research between universities and relevant Institutions or agencies are very imperative.

e) A cooperative effort of public health agencies, physicians, veterinarians, scientists, the food industry, regulatory agencies, research institutes and consumers is needed to control the organism.

f) Listeriosis is a disease of economic importance and, should be made a reportable disease in man and animals.

g) Differential diagnoses techniques for its isolation, identification and characterization is very necessary.

h) Susceptible individuals(pregnant women, AIDS, cancer and diabetic patients should be aware of *L. monocytogenes* in ready-to-eat foods and other farm products that do not require cooking before consumption.

i) Nigerian Government agencies like National Food and Drug Administration (NAFDAC) and Federal Environmental Protection Agency (FEPA) should

include *L. monocytogenes* as reportable pathogen of public health importance in food and environmental hygiene. *L. monocytogenes* should be foodborne organism of interest when checking the microbiology quality of food and other confectionaries meant for human consumption in Nigeria.

j) Good sanitary measures among individuals, homes, food industries and adequate disposal of human and animal wastes (faeces) will enhance in curbing the menace of listeriosis is the Nigerian population/community.

k) Research on the period of viability and seasonal variability of the organisms in the environment is recommended before the application of these wastes on agricultural farm land.

Some publications derived from this study are shown in APPENDIX C

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APPENDIX A

CHEMICALS, MEDIA AND REAGENTS USED

Chemical Composition of Pre- Enrichment Broth

0.1% Tryptone Soy Broth (Oxoid CM129)

Formula per litre

Pancreatic Digest of soybean meal (Oxiod L44)	3.0g
Pancreatic Digest of Caesarea (Oxoid L42)	17.g
Sodium Chloride	5.0g
Debasic potassum phosphate	2.5g
Dextrose	2.5g
pH	7.3
Distilled water	1,000ml

Preparation:

30g of the pre-enrichment broth was weighed using Metler balance and dissolved in 100ml of distilled water. This was mixed and distributed 9mls into aliquots universal bottles and sterilized by autoclaving a 121°C for 15minutes and stored at 4°C.

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

Chemical Composition of University of Vermount (UVM) Modified *Listeria* Enrichment Broth (Difco (223-17-2)

Formula per Litre

Bacto trypose	10g
Bacto beef extract	5g
Bacto yeast extract	5g
Sodium chloride	20g
Sodium phosphate dibasic	9.6g
Potassuim phosphate monobasic	1.35g
Bacto aesuclin	1.0g
Nalidixci acid	0.02g
Aciflavine	0.012g
pH	7.2 ± 0.2

Chemical Composition of *Listeria* Selective Agar (Oxford Formulation)

Listeria selective agar base (Biotest 953 064 005)

Formula per Litre

Cohembia 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

Chemical Composition of PALCAM Selective Agar Base (CM0877)

Composition:

Peptone	11.5g
Starch	0.5g
Sodium chloride	2.5g
D+Manitol	5.0g
Ammonium iron (III) citrate	0.25g
Aesculin	0.4g
Glucose	0.25g
Lithium chloride	7.5g
Phenol red	0.04g
Agar agar	6.5g

Chemical Composition of Palcam Selective Supplement (SRO150E)

Polymyxin B-sulphate	5.g
Ceftaxidine	10mg
Acrifletin	2.5g

Chemical Composition of Nalidrixic Acid Sheep Blood Agar

Blood agar base (Merck$^{(R)}$)	40g	
Sheep blood	7%(1.4×50)	70ml
Nalidixic acid	40mg/%	120ml
Distilled water	1,000ml	

Chemical Composition of Normal Saline

Sodium chloride	0.85g
Distilled water	100ml

Composition of Gram's Stain Reagents

Crysal Violet Gram Stain (1Litre)

Crystal violet	20g
Ammonium oxalate	9g
Ethanol (absolute)	95ml
Distilled water	905ml

Lugol's Iodine Solution (1Litre)

Potassium iodide	20g
Iodine	10g
Distilledwater	1,000ml

Acetone-Alcohol Decolourizer (1Litre)

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25ml

Neutral Red (0.1% w/v) 1Litre

Neutral red	1g
Distilled water	1,000ml

Composition of Sheep Blood Agar for Beta Haemolysis

Blood agar base (Merck®)	40g	
Sheep blood	7%	(1.4×50) 70ml
Distilled water	1,000ml	

Composition of Nutrient Agar for Catalase Test

Blood agar base (Merck®)	40g
Distilled water	1,000ml

Chemical Composition of Motility Test Medium

Motility test medium (Difco)	8g
Nutrient Broth (Difco)	2g
Sodium Chloride	0.5g
Distilled water	500mg

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

Peptone	10g
Meat extract	3g
Sodium chloride	5g
Andrade=s indicator	10ml
Distilled water	1000ml
pH	7.2 ± 1

Chemical Composition of Nitrate Broth

Bacto-peptone (DIFCO)	20
Potassium-nitrate	2g
Distilled water	1,000mls
PH	7.0

Chemical Composition of Nitrate Reduction Test Reagents

Solution A:

Sulphanilic Acid	2.8g
Distilled water	250mls
Glacial acetic acid	100mls

Solution B:

Di-methyl-ɑlpha-napthlyamine	2.1mls
Distilled water	250mls
Glacial acetic acid	100mls

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 dstilled 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.

Chemical Composition of Buffer for PCR Gene Amplification(R)

[3]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 $MgCl_2$, 50mMKCl) 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	1litre
pH	8.3

Chemical Composition of Buffer for Agarose Gel

Sucrose	50 %
EDTA	100µmg
Bromophenol blue	0.1 %

[3] Done accoding to manufacturer's procedure.

Chemical Composition of 4% Agarose Gel

Preparation:

4g of Agarose was dissolved in 100ml of 0.5 Tripple 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 Lactophenol Cotton Blue Stain

Phenol crystals	20grams
Lactic acid	20mls
Glycerol	40mls
Distilled water	20mls
Methylene blue	0.075grams.

Chemical Composition of Phosphate Buffered Saline

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

Calculation for Determination of Total Solid Residue in Experimental Water Samples

In this study, for each experimental water sample a volume of 50mls was used. Thus the calculated result was multiplied by two (× 2) to give the weight of the residue in 100ml of experimental water.

To convert to litre, it was multiplied by ten (× 10), since the S.I unit is milligram per litre (mg/l). The final result was multiplied by one thousand (× 1,000) as shown (A.O.A.C., 1990).

Jos North:

9.0719 – 9.0643 = 0.0076 for 50ml volume of water

$$100\text{ml} \Rightarrow 0.0076 \times 2 = 0.0152\text{g}/100\text{ml}$$
$$0.0152\text{g}/100\text{ml to litre} = 0.0152\text{g}/100\text{ml} \times 10$$
$$= 0.152\text{g/l}$$

$$\text{Weight of sample in mg/l} = 0.152\text{g/l} \times 1{,}000$$
$$= 152\text{mg/l}$$

Jos East:

9.0925 – 9.0914 = 0.0011 for 50ml volume of water

$$100\text{ml} \Rightarrow 0.0011 \times 2 = 0.0022\text{g}/100\text{ml}$$
$$0.0022\text{g}/100\text{ml to litre} = 0.0022\text{g}/100\text{ml} \times 10$$
$$= 0.022\text{g/l}$$

$$\text{Weight of sample in mg/l} = 0.022\text{g/l} \times 1{,}000$$
$$= 22\text{mg/l}$$

Jos South:

9.1400 – 9.1360 = 0.0040 for 50ml volume of water

$$100\text{ml} \Rightarrow 0.0040 \times 2 = 0.0080\text{g}/100\text{ml}$$
$$0.0080\text{g}/100\text{ml to litre} = 0.0080\text{g}/100\text{ml} \times 10$$
$$= 0.08\text{g/l}$$

$$\text{Weight of sample in mg/l} = 0.08\text{g/l} \times 1{,}000$$
$$= 80\text{mg/l}$$

Bassa:

9.1032 – 9.0973 = 0.0059 for 50ml volume of water

$$100ml \Rightarrow 0.0059 \times 2 = 0.0118g/100ml$$
$$0.0118g/100ml \text{ to litre} = 0.0118g/100ml \times 10$$
$$= 0.118g/l$$

$$\text{Weight of sample in mg/l} = 0.118g/l \times 1,000$$
$$= 118mg/l$$

Riyom:

9.0791 – 9.0764 = 0.0027 for 50ml volume of water

$$100ml \Rightarrow 0.0027 \times 2 = 0.0054g/100ml$$
$$0.0054g/100ml \text{ to litre} = 0.0054g/100ml \times 10$$
$$= 0.054g/l$$

$$\text{Weight of sample in mg/l} = 0.054g/l \times 1,000$$
$$= 54mg/l$$

Barkin Ladi:

9.1201 – 9.1110 = 0.0091 for 50ml volume of water

$$100ml \Rightarrow 0.0091 \times 2 = 0.0182g/100ml$$
$$0.0182g/100ml \text{ to litre} = 0.0182g/100ml \times 10$$
$$= 0.182g/l$$

$$\text{Weight of sample in mg/l} = 0.182g/l \times 1,000$$
$$= 182mg/l$$

Percentage Moisture Content of the Experimental Samples

Experimental Samples	Weight of Dish Only	Weight of Experimetal Sample used	Weight of Dish and fresh Sample	Weight of Dish and Dry Sample	Weight of moisture	% Weight of moisture	% Wt. of Dry Matter
Cow Dung	45.7528	3.1935	48.9463	48.9779	1.9684	61.64	38.36
Farm Debris	45.4577	3.1935	48.6512	48.5840	0.0672	2.10	97.90
Goat Faeces	36.0815	3.1935	39.2750	38.7465	0.5285	16.55	83.45
Green Vegetation	38.2187	3.1935	41.4122	39.4245	1.9877	62.24	37.76
Human Faeces	44.8192	3.1935	48.0127	46.0099	2.0028	62.71	37.29
Poultry Droppings	44.8190	3.1935	48.0125	46.0091	2.0034	62.73	37.27
Rabbit Faeces	44.8409	3.1935	48.0344	46.4897	1.5447	48.37	51.63
Sheep Faeces	41.2984	3.1935	44.4919	43.2737	1.2182	38.15	61.85
Soil	44.4536	3.1935	47.6471	47.4510	0.1961	6.14	93.86

The Solid Residue Values of the Experimental Water Samples

Local Govt	Weight of Dish + Solid Residue	Weight of Dish Only	Difference	100ml (× 2)	Gram/Litre (× 10)	mg/L (× 1,000)
Barkin Ladi	9.1201	9.1110	0.0091	0.0182	0.182	182
Bassa	9.1032	9.0973	0.0059	0.00118	0.008	118
Jos East	9.0925	9.0914	0.0011	0.0022	0.022	22
Jos North	9.0719	9.0643	0.0076	0.0152	0.152	152
Jos South	9.1400	9.1360	0.0040	0.0080	0.080	80
Riyom	9.0791	9.0764	0.0027	0.0054	0.054	54

APPENDIX B
STATISTICAL ANALYSES

Statistical Analysis of the General Distribution of *Listeria monocytogenes* and other *Listeria* species from Samples Examined

H_0 = The Observation of *Listeria* species among the various samples has no effect on the environment.

Listeria species	SAMPLES										TOTAL	
	#CD	FD	GF	GV	HF	PD	RF	SF	SL	WT		
L. grayi	3	4	2	3	3	2	3	1	5	1	27	
L. inocua	0	3	0	1	0	0	5	1	2	1	13	
L. invanovii	5	3	4	2	2	8	6	7	3	1	38	
L. monocytogenes	10	7	8	7	4	7	11	13	6	5	78	
L. murrayi	1	1	0	1	0	1	1	1	4	1	11	
L. seeligeri	0	2	0	1	1	1	0	0	1	0	6	
L. welshimeri	1	1	1	1	0	2	4	2	1	3	16	
$\sum x$	20	21	15	16	10	18	30	25	22	12	189	=G
$\bar{x}$	2.86	3	2.14	2.29	1.42	2.57	4.29	3.57	3.14	1.71		
S^2	13.2	4.3	8.8	4.8	2.6	6.3	13.2	22.7	3.8	2.9		

#CD=Cow Dung; FD=Farm Debris; GF=Goat Faeces; GV=Green Vegt.; HF=Hunman Faeces; PD=Poultry Droppings; RF=Rabbit Faeces; SF=Sheep Faeces; SL=Soil; WT=Water

SS* $= \sum x^2 - \frac{G^2}{N} = 6^2 + 3^2 + 1^2 + \ldots + 1^2 + 3^2 + 1^2 - \frac{189^2}{7 \times 10}$

= 1,053 – 510.3

SS = 542.7

SST* $= \frac{\sum T^2}{n} - \frac{G^2}{N} = \left(\frac{22^2}{7} + \frac{21^2}{7} + \frac{20^2}{7} + \ldots + \frac{12^2}{7} + \frac{16^2}{7}\right) - \frac{189^2}{70}$

= (69.1429 + 63 + 57.1429 + … + 20.5714 + 36.5714) – 510.3

= 556.9998 – 510.3

= 46.6998

SSB* $= \left(\frac{78^2}{10} + \frac{38^2}{10} + \ldots + \frac{27^2}{10} + \frac{11^2}{10}\right) - \frac{189^2}{70}$

= (608.4 + 144.4 + … + 72.9 + 12.1) – 510.3

= 883.9 – 510.3

= 373.6

SSE* = SS – (SST + SSB)

= 542.7 – (46.6998 + 373.6)
= 122.4002

Summary Table for Analysis of Variance (ANOVA)

Source of Variation	Sum of Squares	Degrees of Freedom	Mean of Squares	F
Treatments	46.6998	9	5.1889	2.2892
Blocks	373.6	6	62.2667	27.4702
Error	122.4002	54	2.2667	
Total	542.7	69		

Looking at the critical values of F distribution at P = 0.05 and $^{9}/_{54}$ and $^{6}/_{54}$ degrees of freedom are 2.058 and 2.274.

Since 2.2892 > 2.058 and 27.4702 > 2.274 this leads to the reject the null hypothesis. The results obtained from the statistical analysis reveals that the occurrence of the Listeria species in the experimental samples is highly significant.

* SS= Sum of square

SST= Sum of square treatment

SSB = Sum of square block

SSE = Sum of square error

Two-Way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Cow Dung Samples Examined

H_0 = The Occurrence of *Listeria* sepecies among the 90 Cow dung samples examined is not significant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	3	0.6000	2.4	5.76	9.6
L. innocua	0	0.2889	–0.2889	0.0835	0.2889
L. ivanovii	5	0.8444	4.1556	17.2690	20.4512
L. monocytogenes	10	1.7333	8.2667	68.3383	39.4267
L. murrayi	1	0.2444	0.7556	0.5709	2.3361
L. seeligeri	0	0.1333	–0.1333	0.0178	0.1333
L. welshimeri	1	0.3555	0.6445	0.4153	1.1684
TOTAL					73.4046

Thus, as the critical value at P = 0.05 and 6 degrees of freedom is still 12.59 and because the obtained value is 73.4046, there will be rejection of H_0 and it is concluded that there is a significant difference with the observation of *Listeria* species in cow dung.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Farm Debris Samples Examined

H_0 = The Occurrence of *Listeria* species among 90 farm debris examined is not significant.

Performing x^2 test of goodness fit

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O - E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	4	0.63	3.37	11.3569	37.4444
L. innocua	3	0.3033	2.6967	7.2722	1.0521
L. ivanovii	3	0.8867	2.1133	4.4660	5.0367
L. monocytogenes	7	1.82	5.18	26.8324	14.7430
L. murrayi	1	0.2567	0.7433	0.5525	2.1523
L. seeligeri	2	0.14	1.85	3.4596	24.7114
L. welshimeri	1	0.3733	0.6267	0.3928	23.9768
TOTAL					71.6723

Considering x^2 at P = 0.05 at six (6) degrees of freedom is 12.59. But as 71.6723 > 12.59, as before, the null hypothesis is rejected. Hence *Listeria* species among farm debris is significant to the environment.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Goat Faecal Samples Examined

H_0 = The Occurrence of *Listeria* species among Goat faecal samples examined is insignificant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O - E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	2	2.8167	–0.8167	0.667	0.2368
L. innocua	0	0.2667	–0.2667	0.0711	0.2667
L. ivanovii	4	0.633	3.367	11.337	17.91
L. monocytogenes	8	1.3	6.1538	37.8692	29.13
L. murrayi	1	0.1833	0.8167	0.667	3.6388
L. seeligeri	0	0.1	–0.1	0.01	0.1
L. welshimeri	1	0.45	0.55	0.3025	0.6722
TOTAL					51.9545

As the 6 degrees of freedom and the critical value of x^2 is 12.59 and because the obtained value of x^2 is 51.9545 is greater than the critical value, the H_0 is rejected. Hence *Listeria* species among goat debris is significant to the environment.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Green Vegetation Samples Examined

H_0 = The Occurrence *Listeria* species among the green vegetation samples is not significant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	3	0.48	2.52	6.3504	13.23
L. innocua	1	0.231	0.769	0.591	2.558
L. ivanovii	2	0.676	1.324	1.753	2.593
L. monocytogenes	7	1.367	5.633	31.737	23.212
L. murrayi	1	0.1856	0.8044	0.6471	3.308
L. seeligeri	1	0.107	0.893	0.797	7.449
L. welshimeri	1	0.284	0.716	0.513	1.806
					54.156

Here, as the 6 degrees of freedom and the critical value of x^2 is 12.59. but the obtained value of x^2 (54.156) is greater than the critical value the H_0. is rejected ,thus presence of *Listeria* species in the observed samples is significant.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Human Faecal Samples Examined

H_0 = The Occurrence *Listeria* species as examined among 90 human faecal samples as observed is not significant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O - E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	2	0.299	1.701	2.8934	9.677
L. innocua	0	0.1443	–0.1443	0.02082	0.1443
L. ivanovii	2	0.4222	1.5778	2.4895	5.8965
L. monocytogenes	4	0.8667	3.1333	9.8176	11.3276
L. murrayi	1	0.1221	0.8779	0.7707	6.3120
L. seeligeri	1	0.0667	0.9333	0.8710	13.0584
L. welshimeri	0	0.1776	–0.1776	0.0315	0.1774
TOTAL					46.5932

For those data x^2 (6) = 12.59, P = 0.05, examination of the results indicated that the small presence of *Listeria* species in the observed samples is significant.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Poultry Dropping Samples Examined

H_0 = The Occurrence *Listeria* species among 90 poultry faecal samples examined is not significant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	2	0.54	1.46	2.1316	3.9474
L. innocua	0	0.26	–0.26	0.0676	0.26
L. ivanovii	5	0.76	4.24	17.9776	23.6547
L. monocytogenes	7	1.56	5.44	29.5936	18.9703
L. murrayi	1	0.22	0.78	0.6084	2.7655
L. seeligeri	1	0.12	0.88	0.7744	6.4533
L. welshimeri	2	0.32	1.68	2.8224	8.82
TOTAL					64.8712

The x^2 value as calculated 64.8712 is greater than its critical value at the degree of freedom of 6 and probability of 0.05 (12.59), so the null (H_0) hypothesis that the occurrence of *Listeria* species in 90 poultry faecal samples is not significant has to be rejected. In view of the rejection of the(H_0) hypothesis, it implies that from the result of statistical analysis, the occurrence of *Listeria* species in the poultry faecal samples is significant.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Rabbit Faecal Samples Examined

H_0 = The Occurrence of *Listeria* species among 90 rabbit faecal samples examined is not significant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O - E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	3	0.9	2.1	4.41	4.80
L. innocua	5	0.4333	4.5667	20.8547	48.1299
L. ivanovii	6	1.267	4.733	22.4012	17.6805
L. monocytogenes	11	2.6	8.4	70.56	27.1385
L. murrayi	1	0.3667	0.6333	0.4011	1.0938
L. seeligeri	0	0.2	–0.2	0.04	0.2
L. welshimeri	4	0.533	3.467	12.02	22.55
TOTAL					121.693

It was observed that x^2 as calculated is 121.1693 is greater than the critical value of x^2 so this has led to the rejection of the hypthesis ((H_0.) that the occurrence of *Listeria* species in the 90 rabbit faecal samples is significant.

Two-Way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Sheep Faecal Samples Examined

H_0 = The Occurrence of *Listeria* species among 90 sheep faecal samples examined is insignificant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O - E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	1	0.75	0.25	0.0625	0.0833
L. innocua	1	0.3611	0.6389	0.4082	1.1304
L. ivanovii	7	1.0556	6.9444	35.3359	33.4747
L. monocytogenes	13	2.1667	10.8333	117.3604	54.1655
L. murrayi	1	0.3056	0.6944	0.4822	1.5779
L. seeligeri	0	0.1667	–0.1667	0.0278	0.1667
L. welshimeri	2	0.444	1.556	2.4211	5.4529
TOTAL					96.0514

As X^2 critical value at dot degree of freedom of 6 and P = 0.05 is 12.59, so the H_0 is rejected since 96.0514 > 12.59 and this shows that *Listeria* species as examined is significant.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Soil Samples Examined

H_0 = The Occurrence of *Listeria* species among 90 soil samples examined is not significant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	1	0.1467	0.8533	0.7286	4.9632
L. innocua	2	0.3178	1.6822	2.8298	8.9043
L. ivanovii	3	0.9298	2.0711	4.2895	4.6178
L. monocytogenes	6	1.9067	4.0933	16.7551	8.7875
L. murrayi	5	0.2689	4.7311	22.3833	83.2402
L. seeligeri	4	0.1467	3.8533	14.8479	101.2127
L. welshimeri	1	0.3911	0.6089	0.3708	0.9481
TOTAL					212.6692

x^2 at P = 0.05 at six (6) degrees of freedom i.e. (n – 1) is 12.59. Since 212.6692 > 12.59, there is rejection of H_0. This implies that *Listeria* species occurrence in is the soil samples is significant to the environment.

Two-way Statistical Analysis of Variance (ANOVA) of *Listeria* Species Occurrence in the 90 Water Samples Examined

H_0 = The Occurrence *Listeria* species as observed among 90 water samples is insignificant.

Listeria species	Observed (O)	Expected (E)	(O – E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
L. grayi	1	0.36	0.64	0.4096	1.1378
L. innocua	1	0.1733	0.8267	0.6834	3.943
L. ivanovii	1	0.504	0.496	0.246	0.4881
L. monocytogenes	5	1.04	3.96	15.682	15.079
L. murrayi	1	0.1467	0.8533	0.7281	4.9632
L. seeligeri	0	0.08	–0.08	0.0064	0.08
L. welshimeri	3	0.21	2.79	7.7841	37.067
TOTAL					62.7581

Because the critical value on 6 degrees of freedom at P = 0.05 is 12.59, the H_0 is rejected and it is concluded that the observed samples of *Listeria* species have significant effect on the environment.

Two-way Statistical Analysis of Variance (ANOVA) of the Relative Efficiecy of the three (3) Solid Culture Media Employed for the Isolation of *Listeria* species

Listeria species	Culture Medium			Total Positive
	OLA	Palcam	NBA	
L. grayi	27	27	25	79
L. innocua	13	13	9	35
L. ivanovii	38	37	27	102
L. monocytogenes	78	78	70	226
L. murrayi	11	11	7	29
L. seeligeri	6	6	4	16
L. welshimeri	16	16	10	42
$\sum x$	189	188	152	529
$\bar{x}$	27	26.857143	23.142857	
S^2	622.5	619.0	533.15	

$$SS* = 78^2 + 38^2 + 6^2 + \ldots + 25^2 + 7^2 - \frac{529^2}{7 \times 3}$$

$$= 24{,}103 - \frac{279{,}841}{21}$$

$$= 24{,}103 - 13{,}325.7619$$

$$SS = 10{,}777.2381$$

$$SST* = \frac{187^2}{7} + \frac{188^2}{7} + \frac{152^2}{7} - \frac{529^2}{21}$$

$$= (5{,}103 + 5{,}049.1429 + 3{,}300.5714) - 13{,}325.7619$$

$$= 13{,}452.7143 - 13{,}325.7619$$

$$= 126.9524$$

$$SSB* = \left(\frac{226^2}{3} + \frac{102^2}{3} + \ldots + \frac{79^2}{3} + \frac{29^2}{3}\right) - 13{,}325.7619$$

$$= (17{,}025.3333 + 3{,}468 + \ldots + 2{,}30.3333 + 280.3333) - 13{,}325.7619$$

$$= 23{,}935.6665 - 13{,}325.7619$$

$$= 10{,}609.9046$$

$$SSE* = SS \;\; (SST + SSB)$$

$$= 10{,}777.2381 - (126.9524 + 10{,}609.9046)$$

$$= 40.3811$$

Smmary Table for Analysis of Variace (ANOVA)

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F
Treatments	126.9524	2	$\frac{126.9524}{2} = 63.4762$	$\frac{63.4762}{3.3651} = 18.8631$
Blocks	10609.9046	6	$\frac{10609.9046}{6} = 1768.3174$	$\frac{1768.3174}{3.3651} = 525.4873$
Error	40.3811	12	3.3651	
Total	10777.2381	20		

So from table of critical values of the F distribution at a p = 0.05, $2/12$ and $6/12$ degrees of freedom, table F are 3.89 and 3.00 respectively.

Since 18.8631 > 3.89 and 525.4873 >3.00 it only implies that there is relative efficiency differences along the different culture media and also along the different *Listeria* species.

To investigate differences between treatments i.e. the different culture media, we use Duncan's multiple range test.

Their means are arranged in descending order.

OLA	Palcam	NBA
27	26.8571	23.1429

Then the standard error = $\sqrt{\frac{S^2}{4}} = \sqrt{\frac{3.3651}{7}} = \sqrt{0.48073} = 0.693$

From Q-table at k, k – 1 with 12 degrees of freedom are,

	K*(3)	K* – 1 (2)
Q*	3.77	3.08
SSR*	2.6126	2.1344

Substracting the smallest mean from the highest and comparing the difference with shortest significance range (SSR) at K= (27 – 23.1429 = 3.8571). Therefore, comparing it with SSR for k-treatments (2.6126), it is greater. Since 3.8571 > 2.6126, it is significant. Also, with the next smallest mean which is (27 – 26.8571 = 0.1429) and comparing it with k – 1 (2) SSR, 0.1429 < 3.08. Since SSR is less than k – 1 (2), it is not significant, hence both OLA and Palcam media are relatively efficient for the detection of *Listeria* species.

* F= Variance ratio

Q= Critical value (from critical statistical Table)

SSR= Shortest significance range

K= Treatment (factors being considered)

APPENDIX C

PUBLICATIONS DERIVED FROM THIS STUDY

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Chukwu, O.O.C., Ehizibolo, O. D., Mustapha, A.I., Chukwu, D.I., Olabode, O. A., Ogbonna, C.I.C. (2006) *Listeria* species in Smoked Fish on the Retail Markets of Jos, Nigeria , *Animal Production Research Advances* Vol.2 No.4, 231-234.

Chukwu, O.O.C, Ogo I. N., Antiabong, F.J., Muhammad, J.M., Ogbonna C.I.C. and Chukwukere, C.S. (2006) Epidemiological evidence of listeriosis in Guinea Pigs fed with Cabbage (*Brassica oleracea*) in Nigeria. *Animal Production Research Advances* Vol.2 No.4, 248-252.

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Chukwu, O.O.C, Ogbonna, C.I.C., Nwankpa, N. D., Muhammed, M.J., Chukwuuedo, A. A., Olabode, O.A., Nwosu, C. I. and Okewole, P.A. (2004). Listeriosis in a pregnant bitch: A case report.*Proceedings of the* 41st *Congress of NVMA* held at NVRI Vom, 22nd-26th Nov, 2004. Pg 30-31.