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PREVALENCE, ANTIMICROBIAL PROFILES, MOLECULAR SEROTYPING AND TOXIGENICITY OF *Listeria monocytogenes* ISOLATED FROM FOOD IN GABORONE, BOTSWANA

By:

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Submitted in fulfillment of the requirements for the degree of MASTERS OF SCIENCE in Microbiology (LIFE SCIENCES) at the UNIVERSITY OF SOUTH AFRICA

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STATEMENT OF ORIGINALITY

I declare that the study titled Prevalence , antimicrobial profiles, molecular serotyping and toxigenicity of *Listeria monocytogenes* isolated from food in Gaborone, Botswana is my origional work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references. It has been submitted and shall not be submitted in any form to any institution of higher learning for the award of any degree.

Signature of Student Date

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DEDICATION

This dissertation is dedicated to my dear wife, Thabea and our beloved daughters; Christanne, Lebo, Girl, Neo and Basetsana who endured the loneliness during my absence, for their encouragement, support and prayers.

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ABSTRACT

Listeria monocytogenes is known to cause epidemic and sporadic cases of listeriosis. The present study investigated its occurrence, antibiotic sensitivity and serotyping of the organism in foods in various retail outlets in Gaborone, Botswana. Food samples were obtained randomly from selected supermarkets and street vendors from 5 geographical areas in Gaborone from May to September 2007. *Listeria monocytogenes* was isolated and positively identified by using morphological and biochemical tests. Furthermore, the organism was identified using multiplex PCR. From a total of 1324 food samples tested 57(4.3 %) were positive for *Listeria monocytogenes*. Out of the 57 isolates, 7 (12.3%), 3 (5.3%), 0 (0.0%), 27 (47.4%) and 20 (35.1%) were isolated from cheese, raw milk, meat (biltong), frozen cabbage and salad (coleslaw). From the 5 geographical areas selected for sampling in this study, Gaborone south recorded the most number 19 (33.3%) of *L. monocytogenes* isolates while Gaborone west recorded the least, 7 (12.3%). Most of the isolates (49%) belonged to serogroups 4a, 4b and 4c. These isolates were found mostly in cabbage. This was followed by serogroups 4b, 4d and 4e which comprised 30% of the isolates. This is in contrast to most studies that have found serotypes 1/2a and 1/2b to be the most common serotypes in food. That serotype 4b was detected in this study was a significant finding, because this is the number one serotype associated with human listeriosis. REP-PCR was used as a typing tool to characterize the *L. monocytogenes* strains. The method showed great promise as all of the *L. monocytogenes* strains were typable using this method, with good correlation between the REP-PCR profiles and the antibiotic resistant profiles. The findings reveal the presence of multi-drug resistant and virulent *L. monocytogenes* serotype 4b in ready to eat food in Gaborone, Botswana and highlight the need for education and training in food safety programmes.

CHAPTER 1

1.0 INTRODUCTION

Listeria monocytogenes causes a very serious illness known as listeriosis. Individuals who are particularly susceptible to this condition are those who are immunocompromised (as in HIV/AIDS infection), pregnant women, newborn babies, and the elderly (Farber and Peterkin, 1991; McLauchlin *et al*., 2004). Although the incidence of listerosis is low, what is significant is that very high fatalities ranging from 20 to 30% have been reported (Mead *et al*., 1999).

L. monocytogenes is widely distributed in nature and has been isolated from a wide array of food products. The organism is considered hazardous in the food industry due to its ability to grow in gas or vacuum-packaged products at refrigeration temperatures (Duffy *et al*., 1994), low water activity (Nolan *et al*., 1992) as well as low pH (Buchanan *et al*., 1993) and all these measures are important in the control of food pathogens. *L. monocytogenes* is also problematic due to its resistance to antibiotics. The first multiresistant strain of *L. monocytogenes* was isolated in France in 1988 (Poyart -Salmeron *et al*., 1990), thereafter *L. monocytogenes* strains resistant to one or more antibiotics have since been isolated (Franco Albuin *et al*., 1994; Charpentier *et al*., 1995).

It has been shown by various studies that listeriosis is a food-mediated illness (Embarek, 1994; Slutsker & Schuchat, 1999). A wide range of foods such as salads, seafoods, meat, and dairy have been implicated in listeriosis (Bell & Kyriakides, 1998; Schlech, 2000), which follows the oral ingestion of the contaminated food (Finlay 2001). Serotyping has been used extensively to characterize *L. monocytogenes* (Wiedmann, 2002; Wagner & Allerberger, 2003). Thirteen *L. monocytogenes* serotypes (serovars) have been characterized by using specific and standardized antisera (Seeliger & Langer, 1979). Although most clinical isolates belong to serovars 1/2a, 1/2b, and 4b, the majority of strains which have caused large

outbreaks were serovar 4b **(**Kathariou, 2000**),** and serovar 1/2a (Jacquet *et al*., 2002; Zhang and Knabel, 2005). Serovar identification by serological tests has remained popular. However, numerous molecular biology methods such as multiplex PCR (Doumith *et al.,* 2004) have come to the fore in the characterization of *L. monocytogenes* serotypes. Even though a recent study (Manani *et al*., 2006) reported the occurrence of *L. monocytogenes* in frozen vegetables in this country, there is little data on the occurrence of this pathogen in foods in Botswana.

1.2 STATEMENT OF THE PROBLEM

Listeria monocytogenes is a well known food-borne pathogen. This pathogen has been implicated in major outbreaks and sporadic cases in different countries (Belle *et al.,* 1992; El-Shamy *et al.,* 1993). Listeriosis is considered a serious health problem due to the severity of symptoms and its high mortality rate. Inspite of the high mortality rate and the increasing resistance of *Listeria monocytogenes* to antibiotics, there are no reference compendium on the prevalence in different foods, distributional profiles across regions, serotypes and toxigenicity of local isolates in Botswana.

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. Therefore the purpose of this study was to determine the prevalence, distribution, toxigenicity, antibiotic resistance, serological and molecular identifications of *L. monocytogenes* isolated from selected foods in Botswana.

1.3 Objectives of the study

1.3.1 Main objective

The objective of this study was to determine the prevalence, antimicrobial resistance, toxigenicity and serotype identification of *L. monocytogenes* isolated from a variety of ready to eat food in Gaborone, Botswana.

1.3.2 Specific objectives

To determine the prevalence of *L. monocytogenes* in different foods such as vegetables, salads, biltong, cheese and raw milk in five selected geographical areas of Gaborone.

- To ascertain the antibiotic susceptibility profiles of isolates in order to provide updated data and guide clinicians and other health care workers on the empiric management of patients.
- To characterize local *L. monocytogenes* isolates by serological and molecular methods

1. 4 Significance of study

This study will provide baseline information on prevalence, predominant serotypes, toxigenicity and antimicrobial resistance profiles of *L. monocytogenes* in various food products across five geographical areas in Gaborone, Botswana. Data on antibiograms will be useful in the empiric management of patients whereas serological and molecular profiles will provide templates for practical epidemiological applications.

CHAPTER 2

2.0 Literature review

The genus *Listeria* includes 6 different species, *L. monocytogenes, L. ivanovii* sub-species, ivanovii, *L. innocua, L. welshimeri, L. seegligeri* and *L. grayi*. Both *L. monocytogenes* and *L. ivanovii* are pathogenic, but only *L .monocytgenes* is associated with humans and animal illness (Rodriguez-Lazaro *et al*., 2004). All these species are psychotrophic and widely spread in the environment. *L. monocytogenes* has indeed emerged as a foodborne pathogen and a contaminant of various foodstuffs (milk and dairy products, meat and meat products, vegetables and seafood). On the other hand *L. innocua* (non pathogenic Listeria) is a major contaminant of vegetable surfaces and equipment or machinery (Aguado *et al*., 2004).

2.1 Classification

In the past, strains were classified to species level using morphological characteristics and biochemical tests (suspect colonies, motility, catalase, hemolysins, CAMP and API *Listeria* identification system). Currently molecular methods that enable the identification of *Listeria* to the species level include; Random Amplified Polymorphic DNA Polymorphism, to discriminate *Listeria monocytogenes* from *Listeria innocua* (in the 16S rRNA genes), Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR) for the detection of *Listeria monocytogenes* and Pulse-field fingerprinting of *Listeria*, for detection of genomic divisions for *L.monocytogenes* and their correlation with serovars. These new molecular methods may also improve the ability to diagnose pregnancy–associated disease and permit the rapid detection and control of *L.monocytogenes* in the food supply chain (Wiedmann *et al.*, 1993).

2.2 Growth conditions

Listeria monocytogenes is a Gram positive, nonspore forming, facultative anaerobe, intracellular pathogen (short rod) which grows between -4 and 50 °C and a pH range of 4.39

to 9.40. *Listeria monocytogenes* can also multiply in blood, cerebrospinal fluid, monocytes, macrophages, leucocytes and T-cells at body temperature of 37 °C (Farber *et al.*, 1991).

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

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

2.4 Listeria disease

Ingestion of food contaminated with *L. monocytogenes* can result in listeriosis; a severe infectious disease characterized by meningoencephalitis, abortion, septicemia and a high fatality rate of 30 % was traced to *Listeria* (Cocolin *et al*., 2002). Listeriosis predominantly affects certain risk groups including pregnant women, newborn babies, elderly people and immunocompromised patients including HIV positive patients. Most healthy individuals experience flu-like symptoms and those at high risk include cancer patients, individuals taking drugs that affect the body's immune system, alcoholics, pregnant women, persons with low stomach acidity and individuals with HIV/AIDS (Rodriguez – Lazaro et *al.*, 2004).

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

The onset time to serious forms of listeriosis ranges from a few days to 3 weeks. The onset time to gastrointestinal symptoms is greater than 12 hours. *Listeria monocytogenes* invade the gastrointestinal epithelium. Once the bacterium enters the host's monocytes, macrophages or polymorphonuclear leukocytes, it is blood-borne (septicemia) and can grow in body cells (Sebelius *et al.,* 1999). *Listeria monocytogenes* produces an exotoxin listeriolysin (LLO) which is a key agent in human neutrophil activation. The stimulation of these phagocytes, however, requires additional listerial virulence factors of which PIcA may play a prominent role (Sibelius *et al.,* 1998).

2.5 Determinants of Virulence in *Listeria*

In addition to well characterised toxin of *L. monocytogenes*, there are other proteins and surface structures that determine the virulence of *L. monocytogenes*. Polymorphonuclear leucocytes (PMN) are essential for resolution of infections with *L. monocytogenes*. Human neutrophils react to extracellular listerial exotoxins by rapid cell activation (Lammerding *et al.,* 1992). Listeriolysin is centrally involved in triggering degranulation and lipid mediator generation. Other factors affecting the pathogenicity of *L. monocytogenes* are; its capacity for intracellular growth, possession of proteins sequestrating iron from ferritin, the presence of catalase and superoxide dismutase, surface components and hemolysin, indicating that it's virulence is multifactorial .The virulence of the organism may be affected by its growth temperature. Growth of *L .monocytogenes* at a reduced temperature (4)˚C increases its virulence intravenously. The hemolysin of *L. monocytogenes* is recognized as a major virulence factor and its secretion is essential for promoting the intracellular growth and T-cell recognition of the organism (Farber *et al.,* 1991).Beside the characterised Listeriolysin-O (LLO) encoded by the *hly gene*, *L. monocytogenes* also produces two other hemolysins; phosphatidylinositol-specific phospholipase C (Pl-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC). Unlike the LLO which lyses host cells by pore formation, these virulence factors act by disrupting the membrane lipids. The bacterium also produces zinc (2+) dependent protease, which acts like an exotoxin. Mutation in the encoding gene (*mpl*) reduces virulence in mice. An operon called *lmBA* encodes a 20kDa protein located on the bacterial surface. The protein, LMaA induces delayed type hypersensitivity and other CMI responses. There are six *Listeria monocytogenes* virulent genes, namely; *prfA*, *pclA, hlyA, mpl, actA*, and *plcB* located together in one virulence gene cluster between the house keeping

gene idh and *prs*. On the other hand *ActA*, the *actA gene* product, is a surface protein required for intercellular movement and cell to cell spread through bacterially induced actin polymerization.

2.7 Epidemiology and distribution of Listeria

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

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

Table 1.Incidence of listeriosis worldwide

Country	Year (No. of cases)	Incidence/ 10^6 population
United States	1986	7.0
United States	Estimated annual Figure (1600)	8.3
Canada	1988 (60)	2.3
Australia (Western)	1989 (13)	7.6
New Zealand	1989(21)	7.0
Belgium	1989 (48)	4.8
Denmark	1987 (27) 1989 (32)	4.7-5.3 6.0
Finland	1989 (29)	5.9
France	1984 (630) 1986 (811) 1989 (416)	11.3 14.7 8.0
Norway	1987 1989 (7)	4.0 1.6
Scotland	1987 (40) 1988 (35) 1989 (29)	7.0 5.7
Sweden	1987 1989 (32)	8.0 3.8
Switzerland	1988 1989 (34)	6.0 5.0
United Kingdom	1988 1989	5.8 4.3
Germany	1989 (14)	5.8
Yugoslavia	1989	3.0

Source: Farber and Peterkin (1991)

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

In a study of the duration of faecal excretion, it was found that of 12 people examined over 16 months, 11 excreted *L. monocytogenes* on one or more occasions : one for 6 months , one for 4 months , three for 3 months , four for 2 months and two for 1 month . However, no one excreted the same serotype of *L. monocytogenes* in the faeces for a consecutive period of longer than 2 months. It is apparent that although shedding patterns tends to be erratic among different individuals, carriers in some cases can shed the organism for long periods. Although among animals the carrier rate is generally considered to be 1 to 5 % (range 1 to 29%), recent studies involving newer methods for isolating *Listeria* species have indicated that much higher carriage rates may also occur(Farber *et al.*, 1991).

L. monocytogenes can be found on poultry carcasses and in poultry processing plants. The prevalence of pathogens in chickens in many countries is well documented but their presence on South African poultry products has not been extensively investigated. Two studies investigating contamination of food available from street vendors in Johanneburg have been reported, but in these only 6 of the samples tested were raw poultry (Mosupye and Von Holly 1999; 2000). Listeria organisms are documented to be zoonotic; one of the sources of infection is the domestic fowl where it could occur as unapparent infection. The carriage of *Listeria monocytogenes* and other *Listeria* in indigenous birds has not been documented in Kenya (Njagi *et al.*, 2004).

Recently, the prevalence of *Listeria* species in various foods, fish and water was investigated and it was found that *L. innocua* was the most common followed by *L. monocytogenes* and the water was contaminated by faeces.Listeriosis may be caused by all 9 serovars (Table 2) of *L. monocytogenes*, however most cases are due to serovars 1/2a, 1/2b and 4b. The major

food-borne outbreaks of listeriosis, as well as the majority of sporadic cases, have been caused by serovar 4b strains. This suggests that serovar 4b may possess unique virulence properties (Buchrieser *et al.*, 2007). However, geographical differences in the global distribution of serotypes apparently exist. *L. monocytogenes* appears to be a normal resident of the intestinal tract in humans, indicating why antibodies to *Listeria* species are commonly found in healthy people (Charpentier *et al.*, 1999).

Table 2. Serovar distribution from human listeriosis cases worldwide

Country (year)	No. (%) of serovar								
	1	1/2a	1/2b	1/2c	4	3a	3b	4b	4c	unknown
Canada (1998)		4(12.9)	13(41.9)		2(6.6)	1(3.2)	9(29)		2(6.6)	
Belgium (1989)		5(10.4)	5(10.4)					37(77.1)		1(2.1)
Finland (1989)		11(42.3)						15(57.7)		
France (1989)		84(20.2)	50(12)					266(63.9)		16(3.8)
Germany (1969-85)		56(18.2)	32(10.8)					196(66.2)		14(4.7)
Scotland (1987-88)		6(8.3)	4(5.6)		7(9.7)			41(56.9)		4(5.6)
Switzerland (1989)		1(3.5)	9(31)					19(65.5)		
UK (1969-1990)		206(15)	140(10)	49(4)	77(6)	15(1)		872(64)		3(0)
Yugoslavia (1989)		2(6.9)	1(3.4)					26(89.7)		
New Zealand (1989)		4(19)	4(19)					12(57.1)		1(4.9)
Argentina (1970-85)		8								
Brazil (1989)		3(25)						9(75)		

Source: Charpentier *et al.*, (1999)

2.8 Antimicrobial resistance in *L. monocytogenes*

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

been recovered from food, the environment and sporadic cases of human listeriosis (Hadorn *et al*., 1993; Charpentier *et al.,* 1995).Antibiotics to which some *L. monocytogenes* strains are resistant include tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim, and rifampicin (Charpentier and Courvalin, 1999).

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

Transfer of resistance between *L. monocytogenes* can occur in the gastrointestinal tract of domestic animals where both species live and where sub-inhibitory levels of tetracycline may be expected. In fact, tetracyclines are the second most commonly used antibiotics worldwide. They are used extensively in animal foodstuffs, especially for poultry, and it is noteworthy that tetracycline resistance was the single most common resistance marker in food-borne *L . monocytogenes* isolated from chicken and turkey,Chopra and Roberts (2001).

Antibiotic resistance in *L. monocytogenes* is reaching an era where virtually all antibiotics will be rendered ineffective because of various mechanisms employed by *L monocytogenes* to counteract the effects of therapeutic agents. *L.monocytogenes* is currently known to form biofilms in utensils and equipment or food processing machinery (Czajka et al., 1993).

2.9 Antibiotic susceptibility testing

Several types of antimicrobial susceptibility testing methods have been devised. The test used most frequently is the disk diffusion procedure (Kirby-Bauer test), in which clinical interpretations are derived from correlations with the reference test. Mueller-Hinton agar is generally the medium of choice for these tests (Abuin *et al.,* 1994). In the Kirrby-Bauer test, the microorganism is spread on the agar surface in order to get a lawn of growth. As soon as

the antibiotic–impregnated disk comes into contact with the moist agar surface, water is absorbed into the filter paper and the antibiotic diffuses into the surrounding medium. The zone size that is observed in disk diffusion has no meaning on itself, but standards are used to derive a correlation between the zone sizes and minimum inhibitory concentrations (MICs) of the test organism (Abuin *et al.*, 1994).

Epsilometer test (E–test) consists of antibiotic-impregnated strips that are placed on the surface of the agar. The antibiotic content of the strip is graded and the concentration is printed linearly along the strip. After incubation MIC is read from the point on the strip where the zone of inhibition passes. In contrast to the disk diffusion test, where the orientation of the disk does not matter, placing the E- test strip upside down on the agar will alter the results (Abuin *et al.*, 1994; Hamilton–Miller *et al.,* 1995).

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

2.9.1 Typing of Listeria

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

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

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

Plasmid typing was recently used in conjunction with DNA fingerprint to confirm a case of cross–infection with *L. monocytogenes*. However,this technique is of less importance since *L .monocytogenes* does not appear to carry plasmid. On the other hand *L. innocua* carry plasmids ranging in size from 3 to 55 MDa (Aguado *et al.,* 2004). Monocine typing has recently been evaluated as a typing tool for *L. monocytgenes*. Although this technique is potentially promising as an epidemiological tool , only 59 and 56 % of serovars 1/2a and 4b were found to be producers of monocines .In one instance a pair of *L. monocytogenes* strains isolated from a mother and a newborn , which could not be phage typed , proved identical by monocine typing (Baloga *et al.,* 1991).

2.9.2 Isolation and identification of L. monocytogenes

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

Currently, newer methods (molecular), such as pulsed field gel electrophoresis that has been used to differentiate *L.monocytogenes* from *L. inocua* (Howard et al., 1992), random amplified polymorphic DNA (RAPD), real time PCR (Wiedmann *et al.,* 1993; Bubert et al., 1999) and restriction endonuclease analysis (REA), have been employed to directly characterize the microorganism without the need for isolation (Gudmundsdottir *et al.,* 2005).

2.9.3 Diagnosis

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

2.10 Detection of Listeria exotoxin (Listeriolysin O)

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

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

In the analysis of genomic DNA of *Listeria* by southern hybridization with *hlyA* probes, all strains were isolated and digested with the restriction endonuclease HindIII. The 0.8-kb BamHI probe that was made up entirely of sequences upstream of the listeriolysin gene was found to hybridize to *L. monocytogenes* strains irrespective of serotype, as well as to the *L. seeligeri* and *L. ivanovii* strains (Sibelius *et al.*, 1998). Other methods that can be employed

to detect listerolysin are; hemolysin assays and polyacrylamide gel electrophoresis, imuno-magnetic beads for listeria and *Listeria* exotoxin detection kits (Sibelius *et al.,* 1998).

2.10.1 Treatment

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

The treatment of choice for listeriosis remains the administration of ampicillin, penicillin G combined with an aminoglycoside. The association of trimethoprim with sulphonamide, such as sulfamethaxazole in co–trimoxazole, is a second choice therapy (Charpentier *et al.,* 1999).

The most active agent in the combination is trimethoprim, which is synergized by sulfamethaxazole. Most isolates from clinical as well foodborne and environmental sources are susceptible to the antibiotics active against gram positive bacteria (Abuin *et al.,* 1994).

2.10.2 Control and prevention

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

CHAPTER 3

3.0 METHODOLOGY

3.1 Experimental Design

Samples were obtained randomly from selected supermarkets and street vendors in 5 Geographical areas of Gaborone (East between Longitude 24°40'0'S -24°41'0'S and Latitude 25°56'0'E-25°57'0'E, West between Longitude 24°38'0'S-24°39'0'S and Latitude 25°52'0'E-25°53'0'E, North between Longitude 24°37'0'S-24°38'0'S and Latitude 25°56'0'E- 25°57'0'E, South between Longitude 24°40'0'S-24°41'0'S and Latitude25°52'0'E-25°53'0'E, and Central Between Longitude 24°39'0'S-24°40'0'S and Latitude 25°54'0'E -25°55'0'E). Samples were; raw vegetables (cabbage) and salads, raw milk, cheese and meat (biltong). Ideally, 250 -300 samples per product were obtained. The sampling procedure was followed as outlined below

3.2 Sampling

Samples were put in separate properly labeled sterile specimen bags and put into a cooler box containing ice packs. New gloves were worn for each sample to avoid cross- contamination between samples from different supermarkets and street vendors. Aseptic technique was followed to avoid contamination during transport of the samples from the supermarkets to the laboratory.

3.3 Homogenisation

Samples were transferred into properly labeled stomacher bags and then homogenised with the Stomacher (Seward 400, Tekmar, and Cincinnati Ohio, USA) set at medium speed.

3.4 Enrichment, culturing and microscopic observation

The homogenised samples were enriched by putting 25g of the sample into 225ml enrichment broth (Mast Diagnostcs DM257) and incubated at 30°C for 48 hours on Innova 4000

Newbrunswick Scientific shaking incubator. A loop full of culture was subcultured after 48 hours onto Listeria Selective Agar plates and then incubated at 37ºC for 24 hours. Modified Listeria Selective Enrichment Supplement (SR206E, Oxoid, Basingstoke, and Hamshire, England) was added to Listeria Agar Base and dark brown colonies with black zones were subcultured on nutrient agar (Oxoid CM001) plates.

A Gram stain was done on suspected colonies from a culture medium (Nutrient Agar). Colonies that were Gram positive short rods were picked subcultured onto Tryptic Soya Agar (Merk, Darmstadt, Germany) slants. After 24 hour incubation, the slants were kept at 4ºC. Other broth cultures were stored in 80% Tryptic Soya Broth and 20% Glycerol (i.e., 750µl Tryptose broth plus 250µl of 20% Glycerol) were put into a 2ml vial and kept at -82˚C for subsequent steps.

3.5.0 Biochemical testing

3.5.1 Catalase test

A catalase test was performed to separate the *L. monocytogenes* from other *Listeria* species. Picking a colony with sterile loop on a slide containing 3% hydrogen peroxide does this. The evolution of gas bubbles (oxygen) indicates a positive test.

3.5.2 Serological confirmation

Seroagglutination was carried out by a slide agglutination technique using commercially prepared *Listeria* spp. antisera (Oxoid Listeria test kit DR1126A, Basingstoke, New Hampshire, England). Agglutination patterns were linked to *Listeria* spp. following manufacturer's instructions.

3.5.3 API Listeria

Isolates that were positive in the serology test were subjected to the API Listeria test (BioMerieux, Paris, France). This is the confirmatory test for the organism and it differentiates *L. monocytogenes* from other *Listeria* species.

3.6 Antibiotic susceptibility testing

Listeria monocytogenes isolates were inoculated into Mueller – Hinton broth. The flasks were incubated at 37°c on shaking incubator (Innova 4000 Newbrunswick Scientific) set at 2000 rpm for 24 hours. A sterile swab was then used to evenly spread 1ml of cell suspension on the surface of Muller – Hinton agar. Antibiotic disks (for Gram – positive organisms), namely; Chloramphenicol (25µg), Erythromycin (5µg),Fusidic acid (10µg),Methicillin (10µg), Novobiocin (5µg),Penicillin G (1unit),Streptomycin (10µg) Tetracycline (25µg), Ampicillin (10µg), Cephalothin (5µg), Gentamicin (10µg), Nitrofurantoin (10µg) and Sulphamethaxozole/trimethoprim (10 µg) were then placed onto the medium with sterile forceps (Kirby-Bauer test).The test consists of antibiotics–impregnated Mastring disks(Davies diagnostics ,Randburg, Johannesburg, South Africa) that are placed on the agar and plate incubated 37◦C for 24 hrs. The zone of inhibition that is observed in disk diffusion has no meaning on itself, but the NCCLS antimicrobial susceptibility testing standards (M2-A6 and M7-A4) were used to derive a correlation between the zone of inhibition and Minimum Inhibitory Concentration (MIC) of the test organism.

3.7 Exotoxin analysis (Listeriolysin O)

Sheep blood agar was used to determine the presence of listeriolysin (LLO) exotoxin in *L.monocytogenes* strains. Hemolysis on sheep blood agar after 24 hours incubation at 37˚C indicated positive results.

3.8 Rhamnose fermentation

All isolates were confirmed using rhamnose fermentation test, by inoculating Oxidative Fermentation (OF) test medium (Atlas et al., 1993; Roberts et al., 2005) containing 0.55mM rhamnose with a stab of cells taken from individual colonies on Tryptic Soya Agar plates. Two tubes per isolate were stabbed; one was overlaid with sterile mineral oil and both were inoculated at 37˚C for 48 hours. Observations were recorded at 24 and 48 hours and reaction was recorded as positive if the media turned yellow in both tubes, indicating the fermentative formation of acid from rhamnose (Roberts *et al*.2006).

3.9.0 Serotype identification by PCR

3.9.1 DNA extraction

L. monocytogenes strains were stored long term in tryptic soy broth (Merck,Darmstadt, Germany) with 20% glycerol at -82˚C. Strains were then recovered by inoculating into tryptose soy broth and were grown overnight at 37 ˚C. Cells were harvested on a bench top centrifuge and genomic DNA extracted using Guanidium Thiocyanate chromosomal technique (Pitcher et al., 1989). 500µl of guanidium thiocyanate solution (60g Guanidium thiocyanate, 20ml 0.5M EDTA pH8,20ml deionized water and 5ml of 10% (w/v) N-Lauryl-Sarcosine Sodium salt, made up to 100ml with deionized water) was added and briefly mixed to lyse the cells. 250µl ice-cold 7.5M ammonium acetate was added, mixed and left on ice for 10 minutes. 500µl Chloroform/ Isopropanol (24:1) were added, mixed and span at 1200g for 10 minutes. 600µl supernatant was transferred to clean Eppendorf tubes and 400µl ice –cold isopropanol was added, mixed by gentle inversion of the Eppendorf tube and span at 6500g for 20 seconds to collect DNA. The pellet was washed three times with 200µl of ice-cold 70% alcohol, span at 6500g for 2 minutes to remove excess Guanidium thiocyanate. The pellet was then air dried at 4˚C for 7 minutes and resuspened in 30µl of TE buffer and kept at 4˚C for subsequent steps.

3.9.2 PCR Amplification

Amplification of serotype specific gene (*hlyA*) product was done by multiplex PCR using reverse and forward primers (D1-Forwad; 5’ CGATATTTTATCTACTTTGTC 3’; D1-Reverse; 5’ TTGCTCCAAAGCAGGGCAT 3’ and D2-Forward; 5’ GCGGAGAAAGCTATCGCA 3’; D2-Reverse; 5’ TTGTTCAAACATAGGGCTA 3’) as described by Borucki and Call (2003).

Reaction mixture was made up to 50µl using the high pure PCR template kit (Fermentas) and Roche PCR core kit reagent according to manufacturer’s instructions. Each reaction consisted of 50pmol of each primer and 50ng of DNA template with 2.5 units of Tag polymerase.

Amplification was carried out using Applied Biosystems GeneAmp 2400 thermocycler. PCR cycling conditions were as follows; 95°C for 3 minutes followed by 25 cycles (with D1 and D2 primers) 72◦c for 1 minute followed by a final step of 72˚C for 10 minutes after cycling was completed. The product size was resolved using electrophoresis through 1.8% agarose gels containing ethidium bromide and visualized on a UV transilluminator. During this experiment laboratory control strain of *L. monocytogenes* were used as a positive control, and included in each group of samples undergoing analysis.

The strains that tested positive with D1 primers were further subjected to PCR using GLT primers (GLT-Forward 5'- AAA GTG AGT TCT TAC GAG ATT T-3' and GLT-Reverse 5'- AAT TAG GAA ATC GAC CTT CT-3'). The PCR reaction conditions were as mentioned above but with a different PCR cycling protocol. Initial denaturation was carried out at 95◦C for 5 minutes followed by 25 cycles of 45◦C for 30 seconds and 72◦C for 1 minute, followed by a final step of 72◦C for 10 minutes after cycling was completed. PCR products were determined using electrophoresis through 1.8% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

3.9.3 Lineage group classification by MAMA – PCR

MAMA primers were used to test strains that tested negative with GLT primers. The high pure PCR template kit (Fermentas) was used according to the manufacturer's instructions. Reaction mixtures contained primers LM4-Forward (5'- CAG TTG CAA GCG CTTGGAGT-3') and LMB-Reverse (5'- GTA AGT CTC CGA GGT TGC AA-3') at a concentration of 50pmoles. MAMA-PCR amplification conditions were as follows; 10 minutes initial denaturation step, followed by 40 cycles of 0.5 minutes at 95◦C, 1 minute at 55◦C and 1 minute at 72◦C, with a final extension step for 10 minutes at 72◦C. Amplification product was electrophoresed on a 1.5% agarose gel containing 0.4µg/ml ethidium bromide at 60 volts for 90 minutes and visualized on a UV transilluminator.

The strains that tested positive with MAMA primers were subjected to PCR using ORF2110 primers (Forward: 5'- AGTGGACAATTGATTGGTGAA-3' and Reverse: 5'- CATCCATCCCTTACTTTGGAC-3') at a concentration of 50 pmoles.ORF2110 - PCR amplification conditions were as follows; initial denaturation step at 94 ˚C for 3 minutes

followed by 35 cycles of 94 ˚C for 0.40 minutes, 53 ˚C for 1 minute and 72 ˚C for 1 minute and one final cycle of 72 ˚C for 7 minutes. Amplification product was electrophoresed on a 1.5% agarose gel containing 0.4µg/ml ethidium bromide at 60 volts for 90 minutes and visualized on a UV transilluminator.

3.9.4 Typing by repetitive element sequence – based PCR

Amplification of REP-PCR products was done using REP IR – I 5'–IIIICGICGICATCIGGC-3' and REP 2-1 5'-ICGICTTATCIGGCCTAC-3' primer pairs as described by Jersek et al, (1999). Reaction mixtures made up of 50µl using the high pure PCR template kit (Fermentas) and Roche PCR core kit reagents were used according to the manufacturer's instructions. Each reaction consisted of 50 pmol of each primer and 50ng of DNA template with 2.5 units of Tag polymerase.

REP – PCR cycling conditions were as follows; An initial denaturation at 95°C for 3 minutes followed by 30 cycles of 90°C for 30 seconds at 40°C for 1 minute , at 72°C for 1 minute and final cycle at 72°C for 8 minutes.
The REP-PCR gene products were resolved into finger printing patterns on a 1.5% agarose gel (Roche) at 60Volts for 1 hour.

Fingerprint patterns were compared for similarity by visual inspection. Fingerprint patterns were considered different if there was a presence or absence of a band at a particular molecular weight. Variations in the brightness of the band, was not considered to constitute a difference.

Statistical analysis

The data collected was analysed using Statistical Package for Social Science (SPSS 15.0). Person correlation was performed in order to show the relationship between zones, supermarkets, street vendors, products and percentage of positive isolate

CHAPTER 4

4.0 RESULTS

4.1 Prevalence of Listeria monocytogenes

From a total of 1324 of the different food samples tested, 57 (4.3%) were positive for *Listeria monocytogenes*. Out of the 57 isolates, 7 (12.4%), 3 (5.3%), 0 (0%), 27 (47.4%) and 20 (35.1%) were isolated from cheese, raw milk, meat (biltong), cabbage and salad (coleslaw) respectively. *Listeria monocytogenes* was found in all food products except biltong. Figure 4.1 shows that among the food products, the organism was frequently isolated from frozen cabbage (10.11%), while raw milk recorded the least number of isolates (1.08%). The difference in the occurrence of the organism in the foods tested was statistically significant ($p<0.01$; $p=0.000$).

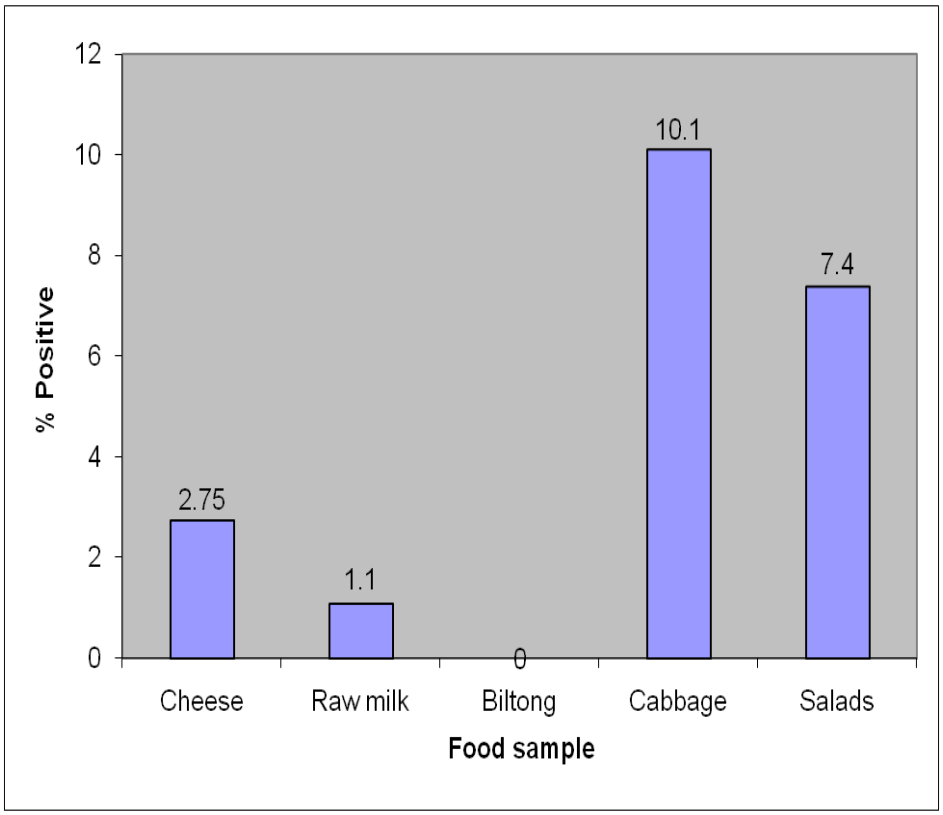

Figure 4.1: Prevalence of *L. monocytogenes* in selected foods in Botswana

4.1.1 Geographical distribution of *L.monocytogenes*

Of the geographical zones sampled in this study, Gaborone south recorded the most number (33.33%) whilst Gaborone west recorded the least (12.28%) with Gaborone east and north recording slightly higher than Gaborone west (Figure 4.2). The prevalence of the organism in the zones sampled was significant ($p<0.01$; $p=0.000$).

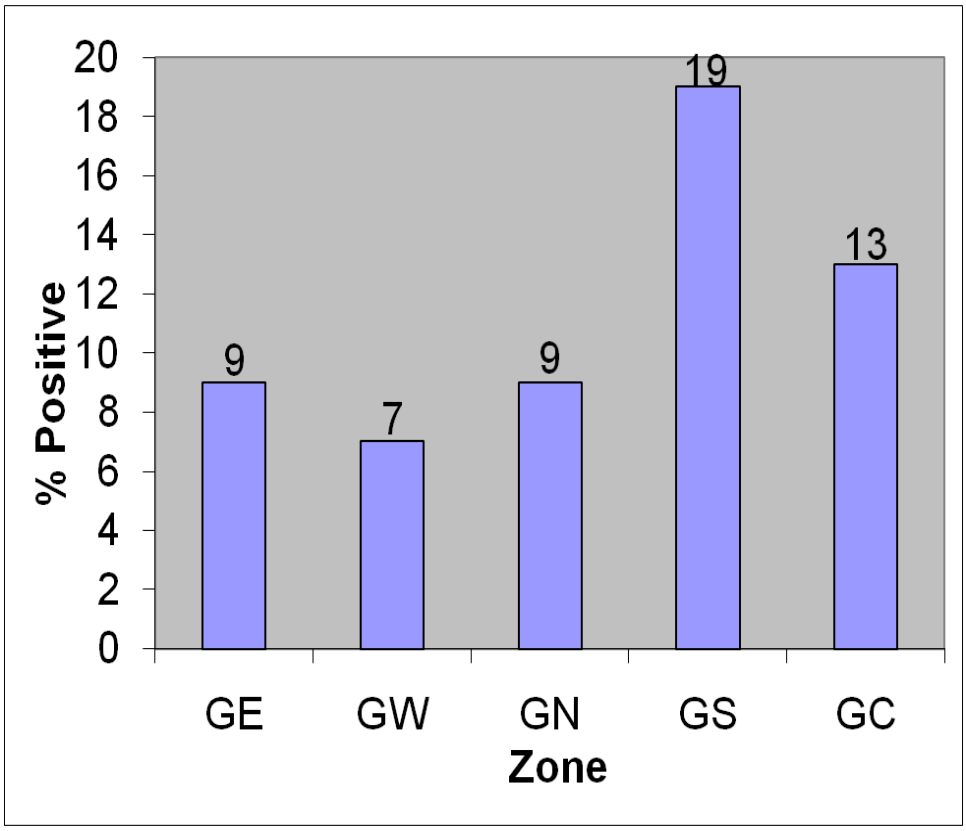

Figure 4.2: Prevalence of *L. monocytogenes* in 5 geographical areas of Gaborone

GE: Gaborone East

GW: Gaborone West

GN: Gaborone North

GS: Gaborone South

GC: Gaborone Central

4.2 Exotoxin analysis (Listeriolysin O)

Isolates of *Listeria monocytogenes* were subjected to the hemolysis test on sheep blood agar. All isolates were β-hemolytic, showing a greenish coloring as depicted in Figure 4.3, albeit in varying degrees.

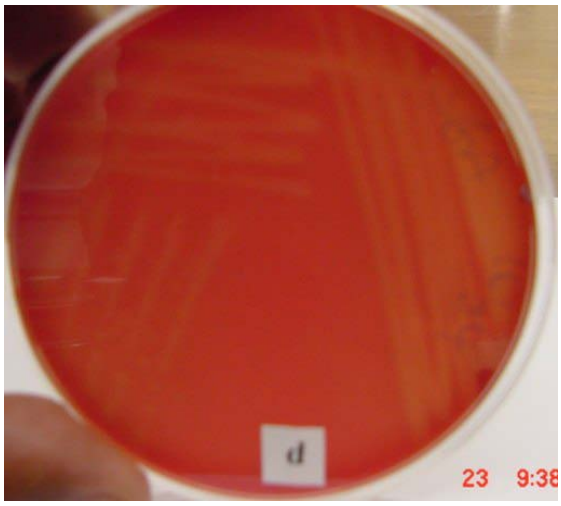

Figure 4.3: ß-haemolysis of *L monocytogenes* on sheep blood agar

4.3 Antimicrobial susceptibility testing of *L.monocytogenes*

Antimicrobial susceptibility testing was performed on all the 57 confirmed *L. monocytogenes* isolates. Of these isolates, 31 (54.39%) were found to be resistant to one or more antibiotics. Resistance to penicillin G, sulphamethaxozole/trimethoprim, chloramphenicol, and tetracycline was encountered in 42.11%, 29.82%, 28.30%, and 22.81% of all the positive isolates respectively (Table 4.1).

Antibiotic resistance was not encountered for fusidic acid, erythromycin, methicillin, ampicillin and cephalothin. In total, 15 different antibiotic resistance patterns were found from the positive isolates. The highest diversity of resistance patterns was found in cabbage and salads. From the resistance patterns, only one pattern (PG, T) was common among all the food products that tested positive for *Listeria monocytogenes* (Table 4.3). Otherwise, the rest of the resistant patterns were unique or peculiar to the different food products that were tested.

Table 4.1: Susceptibility of *L. monocytogenes* to 13 antimicrobial agents

Antibiotic	(%) Sensitive	No. Intermediate	(%) Resistant
Chloramphenicol	71.70	-	28.30
Fusidic acid	100	-	-
Erythromycin	100	-	-
Methicillin	100	-	-
Novobicin	85.96	-	14.04
Penicillin G	54.79	5.0	42.11
Streptomycin	80.70	-	19.30
Tetracycline	67.84	16.0	22.81
Ampicillin	100	-	-
Cephalothin	100	-	-
Sulphamethaxozole/trimethoprim	70.18	-	29.82
Gentamicin	84.21	-	15.79
Nitrofurantoin	92.98	-	7.02

4.4 Serogroup identification by Polymerase Chain Reaction (PCR)

Serogroup identification by multiplex PCR using primer pairs D1 and D2 amplified a PCR product of 214 bp for the entire strains analyzed (See fig 4.4). The PCR product of this size suggests serotypes belonging to phylogenetic lineage of division I and III, which comprise serogroups 4a, 1/2b, 3b, 4b, 4c,4d and 4e.

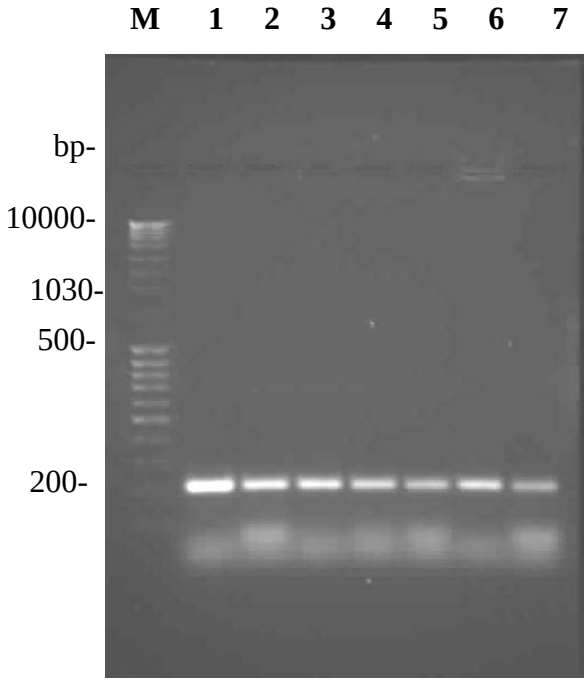

Figure 4.4*:* Genomic DNA from *Listeria monocytogenes* strains subjected to multiplex PCR with primer pairs for D1 and D2.Showing the 214bp PCR product (lanes 1-7).Lane M, MassRuler ^{TM} SM0403 (Fermentas).

To differentiate 1/2b and 3b serotypes from the rest of the members in division I, the strains that tested positive with D1 primers were subjected to PCR using GLT primers. Only 8 (14.04%) strains gave the expected PCR product size of 483bp (Fig 4.5). Unspecific priming was also evident with some cases as shown in lane 5 of Fig 4.5

M 1 2 3 4 5 6 7

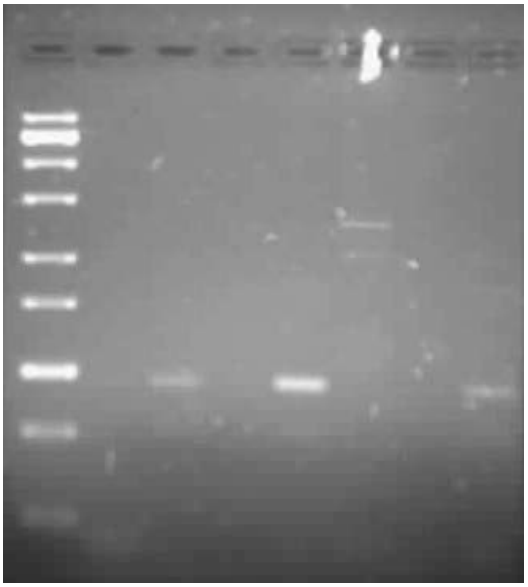

Fig 4.5 .Genomic DNA from *Listeria monocytogenes* strains subjected to PCR with GLT primers. Lane 2, 3 and 7 show isolates with the expected 483bp PCR product.Lane M, ZipRuler ^{TM} SM1378 (Fermentas).

Strains that did not give the expected PCR product size with GLT primers were assumed to belong to either division I or division III. To identify which isolates belonged to serotype 4b the isolates which did not give a PCR product with GLT primers were again subjected to PCR with primers specific to ORF2110. Thirteen of the 57 isolates (22.8%) gave a 597bp amplicon when the PCR product was resolved on a 1, 5% agarose gel (Fig 4.6).

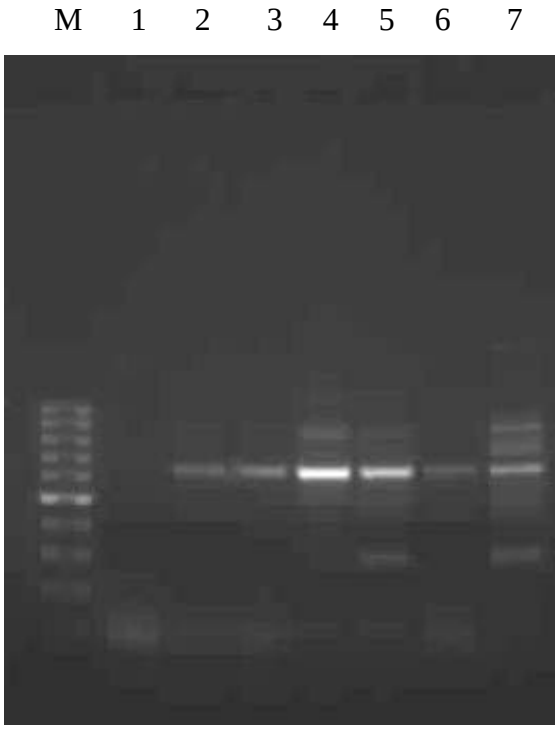

Fig 4.6. Genomic DNA from *Listeria* monocytogenes strains subjected to PCR with primers specific to ORF 2110. Lanes 4 and 5 show strains with the expected 596bp PCR product.Lane M, GeneRuler ^{TM} SM1148 (Fermentas).

Furthermore the strains that were GLT negative were subjected to PCR using MAMA-C primers to identify isolates belonging to division III. From the 43 isolates that were negative for GLT primers, all but one isolate gave a 268bp product with MAMA-C specific primers, indicating that all the isolates belonged to Division III (See fig 4.7). All 13(26.53%) MAMA-PCR positive isolates were positive for ORF2110 and rhamnose fermentation test that confirmed the division III strain.

Fig 4.7 Genomic DNA from *Listeria monocytogenes* strains subjected to PCR with MAMA-C primers. Lanes 2 to 6 show the expected 268bp PCR product. Lane M, GeneRuler^{TM} SM 1148

Serogroup identification using PCR found that most isolates (49%) belonged to serogroups 4a, 4b and 4c. These isolates were found mostly in cabbage and salads. This was followed by isolates in serogroups 4b, 4d and 4e which comprised 30% of the isolates. Isolates within this group appeared in all food types except salads. Isolates with serogroups 1/2b and 3b were rare with four isolates appearing in salads and one isolate being picked up in cheese and milk respectively (see table 4.2). Isolates belonging to division II were not detected at all because no isolates were positive for PCR using D2 specific primers. Four isolates in salads (S10, S31, S200, S258) and two (V208, V225) in cabbage were found to belong to division I, but could not be characterized further into serogroups because they proved to be negative for PCR serogroup identification using GLT and ORF2110 specific primers.

Table 4.2 Serotype identification of *L.monocytogenes* by PCR

Food type	Strains	Serotyping by PCR				Serogroup
		D1	GLT	ORF2110	MAMA-C	
Cheese	C6	+	+	-	-	1/2b and 3b
	C31,C39,C54,C67,C75,C243	+	-	+	+	4b, 4d and 4e
Milk	RM16	+	+	-	-	1/2b and 3b
	RM111,RM117	+	-	+	+	4b, 4d and 4e
Cabbage	V1,V61,V62,V63,V92,V106,V113	+	-	-	+	4a, 4b and 4c
	V121,V129,V131,V148,V157,V189					
	V243,V259,V262					
	V4,V5,V43,V78,V97,V166,V231	+	-	+	+	4b, 4d and 4e
	V238,V250					
	V208,V225	+	-	-	-	ND
Salad	S7,S43,S44,S55,S77,S81,S88,S90	+	-	-	+	4a, 4b and 4c
	S104,S125,S169,S176					
	S1,S24,S199,S221	+	+	-	-	1/2b and 3b
	S10,S31,S200,S258	+	-	-	-	ND

Key: Cheese (C), Raw Milk (RM), Vegetables (V), Salad (S)

4.5 Typing by repetitive element sequence based PCR

All the *L. monocytogenes* strains were typed using REP-PCR. DNA fingerprints obtained for all isolates had a maximum of five bands ranging from 200bp to 300bp (See figure 4.8 A, B, C and D). Out of the 57 strains typed using REP-PCR, 15 profile groups could be identified. Four profile groups were the most predominant than the other REP-PCR types. Profile C was the most common with 21% prevalence; this was followed by REP-PCR profile A, E and B with 19%, 16%, and 14% respectively. Profile group C had a total of 12 isolates which appeared in all food types except milk (see table 4.3). Profile groups A and E were not present in cheese but were evident in all the other food types. While eight isolates with profile group B were present in all food types except milk. Cabbage had the most diverse REP-PCR Profile types with 13 out of a possible total of 15 profile types appearing in this commodity. Only REP-PCR profile group O and I that were unique to salads were absent. Although there was diversity amongst the isolates, the isolates from cabbage and salad showed relative genetic similarity in banding patterns as seen in Fig 4.8 C and D. An example being that most cabbage isolates had a band at 650bp.

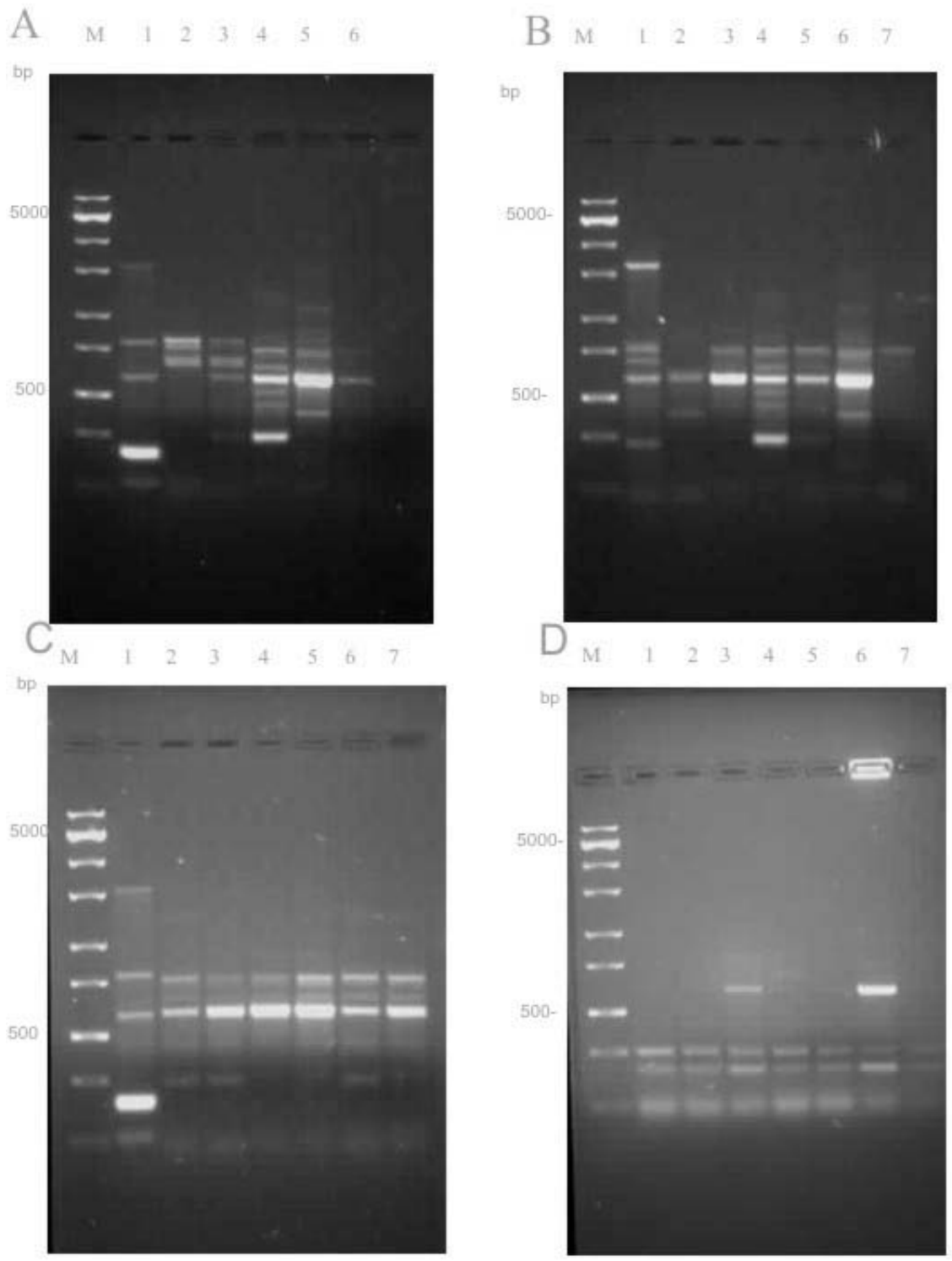

Figure 4.8: REP – PCR fingerprints of *Listeria monocytogenes* isolates (lane 1-7).LaneM, ZipRuler ^{TM} SM1378 (Fermentas).A and B shows the diverse profiles of isolates obtained from cabbage and salads. C and D shows isolates with similar REP-PCR profiles obtained from cabbage and salads respectively.

Table 4.3: Antibiotic resistance and rep profiles of *L.monocytogenes*

Food type	No. of isolates	Antibiotic resistance profile	Resistance strains	Rep-PCR profile
Cheese	7	PG,T	C6	D
		C, PG, NO, S,T	C31	B
		C,PG,NO,S,T	C39,C54,C67,C75,C43	C
Milk	3	PG,T	RM16, RM111	E
			RM117	A
Cabbage	27	PG,T	V4,V5,	D
		S,SX,T	V43,V78	B
		C,PG,SXT	V97,V131,V231,V238,V250	C
		C,PG,NO,T	V1,V63,V92,V106,V113,V189	E
		C,CN,PG,SXT	V131	F
		CN,PG,NO,SXT	V129	M
		C,N,PG,NO,S,T	V148	H
			V208,V225,V243	G
			V106,V166	A
			V259	J
			V262	K
			V121	L
			V157	N
Salad	20	PG,T	S1,S10	C
		NO,PG,T	S7	F
		PG,S,SXT	S24	H
		CN,PG,SXT	S43,S44,S77,S81,S90,S104,S169,S221	A
		C,PG,S,T	S31	I
		C,NI,PG,T	S200	E
		C,NI,PG,NO,S	S55,S88,S125,S176,S199	B
			S258	O

The antibiotics are; Chloramphenicol (C), Gentamycin (CN), Novomycin (NO), Nitrofurantoin (NI), Penicillin G (PG), Streptomycin (S), Sulphamethaxazole/Trimethoprim(SXT),Tetracycline (T)

In most cases there was good correlation between the REP-PCR profiles and the antibiotic resistant profiles within the same food types as well as across food types. In the case of the latter, REP-PCR profile group D was found to be resistant to Penicillin G and tetracycline in both Cheese and Cabbage. Isolates within REP-PCR profile groups C, E A and B were found to fall within corresponding antibiotic resistance profile groups (see fig 4.4). The only major exception was with 10 cabbage isolates with antibiotic resistant profile group C,N,PG,NO,S,T, which all fell into 7 different types of REP-PCR profile groups.

CHAPTER 5

5.0 Discussion

5.1 Prevalence

5.1.1 Prevalence of *L.monocytogenes* in selected foods

In the present study contamination rates of food products with *Listeria monocytogenes* were 2.75%, 1.08%, 0.00%, 10.11%, and 7.41% for cheese, raw milk, biltong, frozen cabbage, and salads (coleslaw), respectively. Cabbage was found to have most isolates (31.58%) and Biltong none.

The non-occurrence of *L. monocytogenes* in biltong is significant in public health because the product is ready-to-eat (RTE) dry meat. Most reports on the occurrence of this foodborne pathogen in meats have tended to concentrate on raw meat, meat mixed with salads and poultry. The absence of the pathogen in biltong is somewhat not surprising because low water activity has been shown to profoundly limit the growth and multiplication of the pathogen in some conditions (Vermeulen *et al.,* 2007). Coupled with this, the meat from this country is exported to the EU markets and the absence of the organism in biltong may be due to the zero tolerance limits in the processing of the meat.

Listeria monocytogenes is known to contaminate milk and milk products such as cheese because of the complex nature of these products (Cimmons, 2001). The incidence rate (2.75%) of *L. monocytogenes* in cheese is less than the 8.2% reported in the United Kingdom (Greenwood *et al.,* 1991) and the 6.4% reported in Germany (Rudol and Scherer, 2001). In Italy, Rudol and Scherer (2001) found the prevalence of *Listeria monocytogenes* in cheese to be as high as 17.4%. In a previous study in Iran (Moshtaghi and Mohamadpour, 2007), the incidence of *L. monocytogenes* in raw milk was found to be 1.6%, which somewhat accord with the 1.08% observed in the present study. Some antimicrobial products such as nisin, reuterin, and the lactoperoxidase system present in milk and milk products are known to have a synergistic bactericidal activity against *Listeria monocytogenes* (Arqeus *et al.,* 2008) hence the lower prevalence figures in the present study among these products than in frozen cabbage and salads. In fact, these antimicrobials are sometimes recommended as natural food

biopreservatives against *L. monocytogenes* as well as other Gram positive organisms (Arques *et al.,* 2008). The prevalence of the organism in cheese is hazardous to consumers because cheese is a ready to eat (RTE) food. Although raw milk is heat treated (pasteurization) before consumption, where the raw milk is processed to dairy products without pasteurization it poses a health risk to the consuming population.

Of public health concern is the occurrence of the microorganism in frozen cabbage (10.11%) and salads (7.41%). A recent study (Little *et al.,* 2007) found a lower proportion (6.0%) of salads to be contaminated with *L. monocytogenes* while another study conducted in the United States of America (Prazak *et al.,* 2002) found an even lower number (2.34%) of cabbage samples to be contaminated. The high incidence of the microorganism in salads and cabbage are alarming because the salads (coleslaw) are ready to eat and the cabbage is sometimes eaten raw when it is mixed with other vegetables to make salads. According to Little *et al.,* (2007), the occurrence of *L. monocytogenes* in pre-packaged mixed salads could result from the original contamination of raw material, cross contamination during processing, packaging or at retail. In chilled foods such as coleslaw and frozen cabbage, temperature is the principal controlling factor for their safety. However, *L. monocytogenes* has been shown to thrive under refrigeration temperatures (Duffy *et al.,* 1994).

5.1.2 Geographical distribution

Among the five geographical areas sampled in the present study, the highest prevalence rate was recorded in Gaborone south (33.33%) whereas Gaborone west recorded the least (12.28%). It is important to note that Gaborone south is characterized by overcrowding and the inhabitants are generally of low economic and educational status. An interesting point to note from this study is that there were no significant differences in the prevalence of *L. monocytogenes* in retail supermarkets and street vendors (See Appendices tables 1&2). A previous study in South Africa (Lues *et al,* 2006) found the microbiological quality of foods served by street vendors to be within acceptable safety limits. In the same study the occurrence of specific microorganisms was thought to be indicative of a degree of ignorance on the part of the food handlers towards proper hygienic practices.

5.2 Exotoxin analysis (Listeriolysin O)

In this study, all the *Listeria monocytogenes* isolates subjected to the β-hemolysis test on sheep blood agar were positive for β – hemolysis. Beta hemolysis is a key characteristic used to differentiate *L. monocytogenes* from non-hemolytic, avirulent *Listeria* species (Cassiday *et al.,* 1990). The hemolytic activity is as a result of the possession by the organism of the virulence factor listeriolysin O (LLO) (Leimeister-Wächter, and Chakraborty, 1989).Encoded by the *hlyA* gene, LLO is important in the intracellular growth of *Listeria monocytogenes* (Gaillard *et al.,* 1986).

5.3 Antibiotic susceptibility testing of *L. monocytogenes*

This study found that resistance profiles to penicillin G, sulphamethaxozole/trimethoprim, chloramphenicol, and tetracycline were encountered in 42.11%, 29.82%, 28.30%, and 22.81% respectively. However, no isolate was resistant to fusidic acid, erythromycin, methicillin, ampicillin and cephalothin. In contrast to the present study, Dhanashree *et al.* (2003) found no strain that was resistant to chloramphenicol in a study in India as did Facinelli et al. (1991) in a survey of Italian meat and dairy products. However, tetracycline resistance has been the most frequently observed phenotype among *L. monocytogenes* strains (Charpentier *et al.,* 1995).

Tetracycline resistance in this study is much higher than the 8.4% in the USA (Zhang *et al.,* 2007). Tetracycline resistance is thought to originate from the use of antibiotic in animal production (Schroeder *et al.,* 2002). A total of 15 antibiotypes were characterized in this study, suggesting a high diversity of antimicrobial resistance of the microorganism in the food products tested. Since the first isolation of a multiresistant strain of *Listeria monocytogenes* in France in 1998 (Poyart Salmeron *et al.,* 1990) multiresistant strains have been extensively isolated.

The data on antimicrobial resistance obtained in this study suggest the importance of a continued surveillance of emerging antimicrobial resistance in *L. monocytogenes* to control the pathogen and ensure effective treatment of human listeriosis. Recent studies (Chou *et al.,* 2006) have reported the possible association between *L. monocytogenes* isolates from fish

products and human listeriosis. However food products contaminated with *L.monocytogenes* remain a potential risk to human health through cross-contamination during food preparation.

5.4 Serogroup identification by Polymerase Chain Reaction (PCR)

In this study serogrouping by PCR suggests serotypes belonging to phylogenetic lineage of division I and III, which comprise serogroups 1/2b, 3b, 4a, 4b, 4c, 4d and 4e. Isolates within this group appeared in all food types except salads. It was found that isolates with 1/2b, and 3b were rare with only four isolates appearing in salad and one isolate being picked up in cheese and milk respectively. This is in contrast to most studies that have found serotypes 1/2a and 1/2b as the most common serotypes in food (Aarnisalo *et al*., 2003), a finding that is also supported in studies by Gilot *et al.* (1996) among foodstuffs in Belgium. A study by Wallace *et al.*, (2003) found serovar 1/2a in 90% of all the *Listeria monocytogenes* isolates tested in food samples. The results obtained from the current study indicated a correlation between certain serotypes and specific food products; serogroups 1/2b and 3b was absent from Cabbage, a food type that had more isolates than other foods. This is in agreement with the results obtained by Vitas and Garcia-Jalon (2004) in a study of fresh and processed foods in Navarra, Spain. In the present study, serotype 4b was detected. This is significant because among the *Listeria monocytogenes* serotypes, serotype 4b has been the number one serotype associated with human listeriosis (Zhang *et al.*, 2007). This serotype has also been detected in foodstuffs in previous studies (Zhang *et al.*, 2007).

One major finding in this study was that isolates belonging to division II were not detected, because no isolates were positive for PCR using D2 specific primers. Division two has serovar 1/2a, a serotype common in food products. Furthermore, four samples belonged to division I, namely; S10, S31, S200 and S258 from salads, V208 and V225 from cabbage, but could not be characterized further into serogroups because they proved to be negative for PCR serogroup identification using GLT and ORF2110 specific primers. This proved to be a major limitation of serotype identification by PCR. One other shortcoming of identification by PCR was that isolates could not definitively be allocated to specific serotypes but only indicated a number of possible serogroups or a division. However, these results are not surprising as the PCR assays used were not based on genes encoding serotype-specific antigens.

5.5 Typing by repetitive element sequence based PCR

Using REP-PCR typing, a significant observation in the results was that most diverse isolates in this study were more common in cabbage and salads than the dairy products. This was to be expected as relatively few *Listeria* isolates were obtained from dairy products. There was a good correlation between the REP-PCR profiles and antibiotic resistant profiles within the same food types as well as across food types. These isolates came from cabbage, salad, cheese and raw milk, with cabbage showing the highest number of profile groups followed by salad. The different REP-PCR types present among food isolates showed relative genetic similarity seen in terms of banding patterns, especially isolates obtained from cabbages and salads. This brings about the possible identification of DNA markers amongst the food types. Therefore REP-PCR typing may prove useful to rapidly determine lineages of epidemiologically linked isolates in investigating outbreak of food-borne disease.

Variations within the sizes of PCR generated fragments using REP-PCR was observed with these study and two others (Wojciech et al., 2004 and Jersek et al., 1998). The amplicons obtained by Wojciech *et al.* (2004) were shorter with sizes ranging between 123 to 735 bp, in comparison to amplicons obtained by Jersek *et al*. with sizes ranging from 298 to 6100 bp. In this study amplicons obtained ranged from 200bp to 300 bp. This study showed a higher amplicon size than Wojciech *et al.* (2004) and recorded less amplicon size than Jersek *et al.*(1998). Though the same primers were used, the reason for such disagreement could be the difference in the DNA polymerase, PCR machines and protocols used in this study.

In this study, REP-PCR was used as a tool to characterize *L. monocytogenes* strains isolated from food. This method showed great possibilities for the typing of *L.monocytogenes.* These data supports previous studies that suggest that REP-PCR can be used as an alternative method for typing *L. monocytogenes*.

5.6 Conclusion

The findings clearly highlight the occurrence of *L. monocytogenes* serotypes 1/2b and 4b among foods served by retailers and street vendors in 5 geographical areas of Gaborone. The presence of this human pathogen in ready-to-eat foods should be considered as having significant public health implications, particularly among the immunocompromised including HIV/AIDS patients who are at greater risk.

L. monocytogenes was resistant to chloramphenicol, penicillin G sulphamethaxozole/trimethoprim and tetracycline, suggesting that the indiscriminate use of these antimicrobial agents for therapeutic purposes in veterinary science and horticulture may lead to the development of antibiotic resistance. Street vendors need to be educated on the dangers of consumption of food contaminated by *L. monocytogenes* .Retailers need to be trained on the importance of maintaining the cold chain,during transportation and storage of food stuffs, considering the occurrence of power disruptions that occur now and then and the high ambient temperatures experienced. Supermarket managers should be encouraged to invest in stand-by generators to serve during periods when power cuts occur.

5.7 Limitations

This study did not aim to investigate the seasonal occurrence of *L.monocytogenes*. Some milk samples were obtained and transported from farms about 15 to 20km to the supermarkets in Gaborone, therefore temperature variation during transportation might have affected some organisms.

5.8 Recommendations

- Investigations of the role of other pathogenic *Listeria* species in Botswana (e.g *Listeria evanovii*) should be undertaken for clinico epidemiological purposes.
- Other food sources such as fish, shrimps, poultry, eggs and vegetables such as lettuce, onion, and broccoli should also be investigated in order to ascertain the frequencies of occurrence, antibiograms and genetic relatedness.
- In the wake of increasing resistance of microorganisms to antibiotics, periodic studies on antibiogram profiles are recommended in order to guide clinicians on empiric management of patients. In addition the activity of medicinal plants against

pathogenic isolates of *Listeria* should be conducted in order to unravel alternative treatment regimes.

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APPENDEX I

Table 1: Summary table1 showing results for the prevalence of Listeria monocytogenes isolated from food sources in different zones in Gaborone

				PRODUCT			
ZONES		**CHEESE**	**RAW MILK**	**BILTONG**	**FROZEN CABBAGE**	**SALADS**	**TOTAL**
GABORONE EAST							
Supermarket A	A	2	0	0	0	1	
Supermarket B	B	1	0	0	0	1	
Supermarket C	C	0	0	0	1	0	
Sreet Vendors		0	1	0	2	0	9
GABORONE WEST							
Supermarket D	D	0	0	0	1	3	
Supermarket E	E	0	0	0	0	0	
Supermarket F	F	1	0	0	2	0	
Street Vendors		0	0	0	0	0	7
GABORONE NORTH							
Supermaket G	G	0	0	0	0	2	
Supermarket H	H	1	0	0	0	1	
Supermarket I	I	1	0	0	1	0	
Street Vendors		0	0	0	3	0	9
GABORONE SOUTH							
Supermarket K	K	1	0	0	5	3	
Supermarket L	L	0	0	0	1	1	
Supermarket M	M	0	0	0	0	2	
Street Vendors		0	2	0	4	0	19
GABORONE CENTRAL							
Supermarket N	N	0	0	0	3	1	
Supermarket O	O	0	0	0	3	3	
Supermarket P	P	0	0	0	0	2	
Street Vendors		0	0	0	1	0	13
TOTAL NUMBER OF POSITIVES		**7**	**3**	**0**	**27**	**20**	**57**
TOTAL NUMBER OF SAMPLES TESTED		**255**	**279**	**279**	**267**	**270**	**1324**
TOTAL NUMBER OF POSITIVES IN %		**2.75**	**1.1**	**0**	**10.1**	**7.4**	**4.3**

Map of Gaborone showing different zones from which samples were obtained

Table 2.of Correlations.spo

Correlations

		Gaborone zones	Gab supmkts	Food product	Positiv isolates
Gaborone zones	Pearson Correlation	1	.000	1.000(**)	.324(**)
	Sig. (2-tailed)		1.000	.000	.001
	N	100	100	100	100
Gab supmkts	Pearson Correlation	.000	1	.000	-.012
	Sig. (2-tailed)	1.000		1.000	.905
	N	100	100	100	100
Food product	Pearson Correlation	1.000(**)	.000	1	.324(**)
	Sig. (2-tailed)	.000	1.000		.001
	N	100	100	100	100
Positiv isolates	Pearson Correlation	.324(**)	-.012	.324(**)	1
	Sig. (2-tailed)	.001	.905	.001	
	N	100	100	100	100

** Correlation is significant at the 0.01 level (2-tailed).

Table3. Summary for ß-haemolysis data from sheep blood agar

	Food product				
Degree of reaction	Cheese	Raw milk	Biltong	Frozen cabbage Salad	
Slighly positive (+/-)	4	0	0	10	9
Positive (+)	3	2	0	9	5
Highly positive (++)	0	1	0	6	4
Strong positive (+++)	0	0	0	2	2
Total number of positive	7	3	0	27	20
Total number % positive	100	100	0	100	100

Table4. Summary of Molecular serotyping of *L.monocytogenes* by PCR

Sample	Primer D1	Primer D2	Primer GLT	Primer MAMA	Serotype ORF2110	Rhamnose Utilization
C6	+	-	+	-	-	-
C31	+	-	-	+	-	+
C39	+	-	-	+	+	+
C54	+	-	-	+	+	+
C67	+	-	-	+	+	+
C75	+	-	-	+	-	+
C243	+	-	-	+	-	+
RM16	+	-	+	-	-	-
RM111	+	-	-	+	+	+
RM117	+	-	-	+	+	+
V1	+	-	-	+	-	+
V4	+	-	-	+	+	+
V5	+	-	-	+	+	+
V43	+	-	-	+	+	+
V61	+	-	-	+	-	-
V62	+	-	-	+	-	+
V63	+	-	-	+	-	+
V78	+	-	-	+	+	+
V92	+	-	-	+	-	+
V97	+	-	-	+	+	+
V106	+	-	-	+	-	+
V113	+	-	-	+	-	+
V121	+	-	-	+	-	+
V129	+	-	-	+	-	+
V131	+	-	-	+	+	+
V148	+	-	-	+	-	+
V157	+	-	-	+	-	+
V166	+	-	-	+	+	+
V189	+	-	-	+	+	+
V208	+	-	+	-	-	-

V225	+	-	+	-	-	-
V231	+	-	-	-	-	-
V238	+	-	-	-	-	-
V243	+	-	-	+	-	+
V250	+	-	-	+	+	+
V259	+	-	-	+	-	+
V262	+	-	-	+	-	+
S1	+	-	+	-	-	-
S7	+	-	-	+	-	+
S10	+	-	-	-	-	-
S24	+	-	+	-	-	-
S31	+	-	-	-	-	-
S43	+	-	-	+	-	+
S44	+	-	-	+	-	+
S55	+	-	-	+	-	+
S77	+	-	-	+	-	+
S81	+	-	-	+	-	+
S88	+	-	-	+	-	+
S90	+	-	-	+	-	+
S104	+	-	-	+	-	+
S125	+	-	-	+	-	+
S169	+	-	-	+	-	+
S176	+	-	-	+	-	+
S199	+	-	+	-	-	-
S200	+	-	-	-	-	-
S221	+	-	+	-	-	-
S258	+	-	-	-	-	-
ATCC19115(4b)	+	-	-	+	-	+

APPENDIX II

Publications

1. Morobe I.C., Obi C.L., Nyila M.A., Matsheka M.I., Gashe B.A.; Prevalence and antimicrobial resistance profiles of *Listeria monocytogenes* isolated from food in Gaborone, Botswana. (Accepted and resubmitted for publication, April 2008: *African Journal of Biotechnology*).

2. Morobe I.C., Obi C.L., Nyila M.A., Matsheka M.I., Gashe B.A.; Molecular serotyping and toxigenicity of *Listeria monocytogenes* isolated from food in Gaborone, Botswana. (To be submitted for publication)

Presentations at conferences

1. Prevalence and antimicrobial resistance profiles of *Listeria monocytogens* isolated from various foods in Gaborone, Botswana (September 2007, Mukleneuk Campus, UNISA, Pretoria).

2. Molecular serotyping and toxigenicity of *Listeria monocytogenes* isolated from various foods in Gaborone, Botswana (September 2008, Mukleneuk Campus,UNISA, Pretoria).

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