# The implementation of sub-typing techniques to determine the diversity of *L. monocytogenes* strains adapted to the food processing environment and their association with human listeriosis cases

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## DECLARATION

I, the undersigned, declare that **'The implementation of sub-typing techniques to determine the diversity of *L. monocytogenes* strains adapted to the food processing environment and their association with human listeriosis cases'** is my own work and that all sources I have used or quoted have been indicated and acknowledged by means of complete references.



Miss Diane Rip

Signature……

Date……

This thesis represents a compilation of articles where each chapter is an individual entity and some repetition between chapters has been unavoidable.

The style of this thesis is in accordance with that of the *Journal of Applied Microbiology*

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# The implementation of sub-typing techniques to determine the diversity of *L. monocytogenes* strains adapted to the food processing environment and their association with human listeriosis cases

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## LIST OF ABBREVIATIONS

- Act A — Actin Polymerizing protein A
- API — analytical profile index
- $a_w$ — water activity
- β — beta
- bp — base pair
- BPW — buffered peptone water
- BSA — bovine serum albumin
- C — Cytosine
- CCP — critical control point
- CFSAN — Center for Food Safety and Applied Nutrition
- cfu $g^{-1}$ — colony forming units per gram
- cfu $ml^{-1}$ — colony forming units per milliliter
- dATP — deoxyadeninetriphospate
- dCTP — deoxycytosinetriphosphate
- dGTP — deoxyguaninetriphosphate
- dNTP — deoxynucleotidetriphosphate
- dTTP — deoxythyminetriphosphate
- *et al.* — and others
- FB — Fraser broth
- FDA — Food and Drug Administration
- FSIS — Food Safety and Inspection Service
- G — Guanine
- h — hour
- HACCP — Hazard Analysis and Critical Control Points
- Inl — Internalin
- kDa — kilo Dalton

- LEB Listeria enrichment broth
- LLO listeriolysin O
- MAMA-PCR mismatch amplification mutation assay-PCR
- max. maximum
- min minutes
- min. minimum
- μl microliter
- μM micromolar
- mM millimolar
- PCR polymerase chain reaction
- PFGE pulsed field gel electrophoresis
- PLC's Phospholipase C
- ppm parts per million
- PrfA positive regulatory factor A
- RFLP restriction fragment length polymorphism
- rpm revolutions per minute
- RTE ready to eat
- s seconds
- SNP single nucleotide polymorphism
- spp. species
- *Taq* *Thermus aquaticus*
- USDA United States Department of Agriculture
- US/DHHS United States Department of Health and Human Services
- USFDA United States Food and Drug Administration
- < less than
- 1° primary
- 2° secondary

## LIST OF CONFERENCE OUTPUTS EMANATING FROM THE WORK REPORTED HEREIN

**Rip, D** and Gouws, P. A. (2009) Epidemiology of the serotypes of *Listeria monocytogenes* in the food supply in and around the Western Cape and their association with documented clinical cases. *Campylobacter, Helicobacter* and Related Organisms (CHRO), Niigata Japan

**Rip, D** and Gouws, P. A. (2010) Prevalence and distribution of the serotypes of *L. monocytogenes* in the food supply in the Western Cape (South Africa) and their association with documented clinical cases. 15th World Congress of Food Science and Technology: The International Union of Food Science and Technology (IUFoST), Cape Town International Convention Centre (CTICC)



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# CHAPTER 1

## 1.1 INTRODUCTION

*Listeria monocytogenes* has been established as a food-borne pathogen since the early 1980s and has become a big concern for the food industry and Public Health authorities (Doyle 2001; Oliveira *et al*. 2003; Capita *et al*. 2005; Conly and Johnston 2008). It is a Gram-positive, opportunistic facultative intracellular bacterium which is frequently present in nature and may be found in any food environment (Liu 2006; Chen *et al*. 2007; Conly and Johnston 2008). Of the six species of *Listeria*, *L. monocytogenes* is the only one capable of causing listeriosis, a severe food-borne illness in humans (de Vasconcelos *et al*. 2008). For the average healthy person, although the incidence of infection is low, symptoms of febrile gastroenteritis may be presented (Gianfranceschi *et al*. 2007; Kersting *et al*. 2010). In immunocompromised individuals however, the hospitalization and mortality rates are amongst the highest for pathogenic organisms (Tran and Kathariou 2002; Lin *et al*. 2006; Schuppler and Loessner 2010). Illnesses such as septicaemia and central nervous system infections may also occur in these individuals (Roberts *et al*. 2006; Schuppler and Loessner 2010). Pregnant women and their fetus are also largely at risk where pre-term delivery and birth defects may occur as a result of listeriosis (Doyle 2001; Garrido *et al*. 2008). The epidemiological surveillance systems for the reporting of listeriosis are poor as it is a non-notifiable disease in many countries.

Therefore, the incidence of infection that is regarded as low must be reconsidered (Mammina *et al*. 2009; Pinto *et al*. 2010).

*Listeria monocytogenes* can reproduce in a wide variety of reservoirs within food processing plants, thereby contaminating the food which then poses a risk for food-borne illness. It can be transmitted from infected animals to humans and also through the consumption of foods from animal origin (Kalender 2003; Kersting *et al*. 2010). Animals are infected by *Listeria* spp. found in the environment; the organism is then transmitted through the blood, milk and excrement of the animal back into the environment where manure, soil, feed and water can become contaminated again (Akpolat *et al*. 2004; Kersting *et al*. 2010). Poultry products and ready-to-eat (RTE) food that support the growth of *L. monocytogenes*, including soft cheeses, unpasteurized milk, hotdogs, deli meats, vegetables and fruits have been linked to cases of listeriosis (Rørvik *et al*. 2003; Chen *et al*. 2007; Conly and Johnston 2008; Ford 2010; Kersting *et al*. 2010). Regardless of HACCP systems that are in place in the food processing plants, listeriosis outbreaks still occur as a result of the ingestion of these food products.



Serotyping, based on the serological reaction between somatic (O) and flagellar (H) antigens and their corresponding sera, has identified 13 *L. monocytogenes* serotypes (Nadon *et al*. 2001; Wiedmann 2002; Kérouanton *et al.* 2010). Of the 13 serotypes of *L. monocytogenes*, 1/2a, 1/2b and 4b are responsible for more than 95% of listeriosis infections in humans (Mereghetti *et al.* 2002; Moorhead *et al*. 2003; Borucki *et al*. 2004;

de Vasconcelos *et al*. 2008). *L. monocytogenes* serotypes 1/2a and 1/2b are mainly associated and isolated sporadically from food and 4b is responsible for the major human epidemic cases (Gilbreth *et al*. 2005). *L. monocytogenes* serotypes 1/2a and 1/2b are also responsible for sporadic cases of human illness (Wiedmann 2002).

Alternative sub-typing methods to serotyping have been investigated since this technique has its limitations, one of which is that only three serotypes are associated with human listeriosis resulting in the value of serotyping being limited (Nadon *et al*. 2001; Gilbreth *et al*. 2005; Liu 2006). Serotyping is not only labour intensive but reagents are expensive and high quality antisera are required (Doumith *et al*. 2004; van Belkum *et al*. 2007). To improve sub-typing discrimination, it is advisable that two or more sub-typing techniques be used for the epidemiological investigation of *L. monocytogenes* serotypes involved in listeriosis (Wiedmann 2002; Liu 2006). Serotyping may play an accessory role as a sub-typing technique for these investigations (van Belkum *et al*. 2007; Garrido *et al*. 2008). As a result, alternative molecular approaches are desired to overcome the limitations of serotyping and to attain better discriminations between strains (Nadon *et al*. 2001). Pulsed field gel electrophoresis (PFGE) has become the regular sub-typing method for investigating listeriosis outbreaks and differentiating between the isolates involved in food-borne outbreaks (Kérouanton *et al*. 2010). It is considered the gold standard for sub-typing of most bacterial pathogens (Wiedmann 2002; Chen *et al*. 2007; Rivoal *et al*. 2010).

Both DNA fragment-based and sequence-based molecular sub-typing methods have been used to investigate listeriosis outbreaks (Revazishvili *et al*. 2004). According to Chen *et al.* (2005), an important function of molecular sub-typing is to accurately identify the clonal relationship among isolates in order to identify outbreaks sooner and also to identify the routes of transmission. This bears epidemiological relevance, in that isolates that are associated with an epidemic/outbreak can be clustered and excluded from those that are not associated to the epidemic/outbreak. The pathogenic potential of strains may differ among clonal groups (Jeffers *et al*. 2001). Isolates can be grouped relative to its outbreak potential. The serotypes of *L. monocytogenes* are known to be genetically diverse with differences in virulence potential being displayed by serotypes (Liu 2006); however detailed knowledge of the diversity and evolution of isolates is still deficient. Different *L. monocytogenes* serotypes show host preferences signifying that their interaction with a host may be directed by genetic factors (Jeffers *et al*. 2001). Epidemiological analysis and population genetic studies of *L. monocytogenes* using sub-typing methods have been really vital in improving the understanding of how *L. monocytogenes* is transmitted from animals or the environment through foods to humans (Ducey *et al*. 2007). It is important to be able to differentiate between serovars since it may even be more useful in the future when additional information and knowledge becomes available concerning the relationship between the serovar and its pathogenic and epidemic potential (Borucki *et al*. 2004). It has been determined that some strains of *L. monocytogenes* are more virulent than others (Doyle 2001; Revazishvili *et al*. 2004; Liu 2006; Ward *et al*. 2010) and that low virulence can be attributed to point mutations in a

number of virulent genes (Doyle 2001; Chen *et al*. 2005; Témoin *et al*. 2008). A suggestion by several investigators is that *L. monocytogenes* clinical strains are of one clonal origin (Revazishvili *et al*. 2004).

In South Africa, food-borne diseases are under-reported due to there being a lack of an adequate food-borne surveillance or notification system (Makhoane 2003). Considering the lack of information in Africa concerning listeriosis, the present study aimed to analyze *L. monocytogenes* clinical isolates from the Western Cape region in order to determine the serotypes involved in listeriosis illness. A total of seven clinical human isolates were characterized into lineage groups by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) analysis and further analyzed by PFGE. In total, 262 samples were included in this study for molecular characterization. A total of 172 food, environmental and human clinical samples tested positive for *L. monocytogenes* which included guacamole (112), salad and vegetables (13), seafood (three), environmental (16), meat (17), rooibos tea leaves (one), milk (one), chicken blood (one) (viable and culturable isolate), unknown food type (one) as well as isolates from human listeriosis cases (seven). Of the remaining 90 samples which were representatives of chicken blood, 40 samples were analyzed by PCR and tested positive for the *hly* gene of *L. monocytogenes*; however isolates showed no viability and was not culturable on selective plating media indicating that the *L. monocytogenes* DNA amplified by PCR may have been non-viable or viable but non-culturable cells. A total of one chicken blood sample showed viability by culture based methods.

Widely used methods for phylogenetic studies have characterized the *L. monocytogenes* serotypes into three lineage groups (Jeffers *et al*. 2001; Wiedmann 2002; Ragon *et al*. 2008). Lineage group representation of isolates of *L. monocytogenes* among the samples listed above was determined by PCR-RFLP analysis of a region of the *hly* virulent gene and was also confirmed by mismatch amplification mutation assay (MAMA)-PCR. These techniques characterized isolates as lineage/genotype I (represented by serotypes 1/2b, 3b, 4b, 4d and 4e), lineage II (1/2a, 1/2c, 3a and 3c) or lineage III (4a and 4c). Furthermore, PFGE was employed to determine the diversity of *L. monocytogenes* strains in food, the food processing environment and human listeriosis cases.



The main objectives of this research study were to (i) analyze RTE food products for the presence of *L. monocytogenes* as well as animal isolates and human isolates (ii) determine the distribution and ecology of the serotypes of *L. monocytogenes* in the food processing environment in and around the Western Cape and those involved in human listeriosis cases by (iii) applying molecular tools to genotype a strain, which include RFLP analysis and PFGE. Currently, there are no surveillance reports or epidemiological data for these findings in Africa (Lemes-Marques *et al*. 2007) and this study will form the basis for generating novel information about the distribution and ecology of *L. monocytogenes* strains and lineage groups in food and their association with clinical cases. This study will improve the understanding of the host preferences of isolates directed by genetic links between *L. monocytogenes* strains derived from food and the

strains responsible for human listeriosis. It will also pave the way for more research to be undertaken in the future, especially in Africa.

# CHAPTER 2

## LITERATURE REVIEW

### 2.1 INTRODUCTION

The genus *Listeria* and listeriosis was named after the English pioneer Dr. Joseph Lister (1827-1912). He was an English surgeon who made a huge contribution to the implementation of the principle of antisepsis in surgical procedures and as a result reduced postoperative deaths. Listerine® mouthwash was also named after him (Medicine Index Accessed May 11, 2011).



Phylogenetic analysis of the 16S rRNA gene determined that the genus *Listeria* is a member of the *Clostridium-Bacillus-Lactobacillus* subdivision with *Brochothrix thermosphacta* being the most closely related (Rocourt and Mollaret 1988). The genus *Listeria* comprises 6 characterized species, namely *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. grayi* and *L. welshimeri* (de Vasconcelos *et al*. 2008; Jeyaletchumi *et al*. 2010). This sub-division has proven useful for practical and epidemiological purposes especially problems associated with food-borne listeriosis (Vázquez-Boland *et al*. 2001b; Cocolin *et al*. 2002; Schmid *et al*. 2005; Hain *et al*. 2006). Of these species, *L. monocytogenes* is the only human and animal pathogen capable of causing severe infections like septicemia, meningitis, perinatal infections, encephalitis

and gastroenteritis and has been associated with abortions (Cox *et al*. 1998; Bubert *et al*. 1999; Jeffers *et al*. 2001; Dussurget *et al*. 2004; Ward *et al*. 2004; Schuppler and Loessner 2010). Other than *L. monocytogenes, L. ivanovii* is the only species of the genus considered important as a pathogen infecting animals, particularly sheep (ruminants) (Axelsson and Sorin 1998; Vázquez-Boland *et al*. 2001a; Domínguez-Bernal *et al*. 2006) and causing listeriosis, which is mainly transmitted by the consumption of spoiled silage (Hain *et al*. 2006). It is known to cause spontaneous abortion in sheep and cattle (Koneman *et al*. 2006). The clinical sign or most prominent symptom is where cattle with listeriosis are seen walking in circles; nicknamed 'circling disease' (Leite-Browning 2008).



*Listeria monocytogenes* is a non spore-forming, Gram-positive, chemoorganotrophic, facultative anaerobic rod that causes severe human food-borne disease (Farber and Peterkin 1991; De Cesare *et al*. 2001; Jiang *et al*. 2006) of which incidences in reported cases have increased in the last few decades (Holko *et al*. 2002; Choi and Hong 2003; Mammina *et al*. 2009). Members of the *Listeria* genus are small (0.4-0.5 μm in diameter and 0.5-2.0 μm in length). They have psychrophilic characteristics and outgrow most other bacteria at 4˚C (Schlech 2000) although they have optimal growth at 30˚-37˚C (Pearson and Marth 1990). Peritrichous flagella that are produced at 20˚-25˚C but not at 37˚C make the organism motile (Peel *et al*. 1988; Todar 2008) (Figure 2.1a and Figure 2.1b). They are catalase positive and oxidase negative and display β hemolysin activity, which produce clear zones on blood agar (Pearson and Marth 1990; Farber and Peterkin

1991; Axelsson and Sorin 1998). The ability of *L. monocytogenes* to grow at refrigeration temperature results in its presence being a lethal threat to the food industry e.g. the dairy industry (Cox *et al*. 1998), where soft cheeses and unpasteurized milk are susceptible. The growth of *Listeria*, more importantly, *L. monocytogenes* in a cold environment (refrigeration temperatures) also makes cold-stored food possible vehicles for food-borne listeriosis and a significant threat to the safety of ready-to-eat (RTE) meat products (Zhu *et al*. 2005; Ducey *et al*. 2007).

Listerial infections are dangerous particularly to immuno-compromised individuals, pregnant women, the elderly and newborns (Cox *et al*. 1998; Yde *et al*. 2010). Healthy children and adults occasionally get infected with *Listeria*, but rarely become seriously ill (CDC 2005). The incubation period ranges from one to 90 days with an average time of 30 days (Yde and Genicot 2004; Curtis 2007). The long incubation time makes it difficult to trace the infection (Chen *et al*. 2007; Yde *et al*. 2010). Although the infective dose is unknown, $>10^3$ cfu $g^{-1}$ is considered to be the number that causes illness in healthy individuals (Curtis 2007).



*Listeria monocytogenes* is a ubiquitous organism, isolated from a variety of sources, namely soil (where it leads to contamination of plant material), plant and vegetation sources, RTE foods, water samples and human and animal feces (Bubert *et al*. 1999; Cocolin *et al*. 2002; Nightingale *et al*. 2005; Valero *et al*. 2006; Kérouanton *et al.* 2010). The natural habitat of *Listeria* is considered to be the surface layer of soil, which is rich

in decaying plant matter (Vázquez-Boland *et al*. 2001a; Ford 2010). Large outbreaks of listeriosis have been associated with contaminated foodstuffs including raw vegetables, milk, meat products, various cheeses and seafood, ice cream and chocolate (Boerlin *et al*. 1997; Doyle 2001; Bremer *et al*. 2003; Choi and Hong 2003; Lafarge *et al*. 2004; Rijpens and Herman 2004; Ford 2010). Over the last few years, listeriosis outbreaks have been the leading cause of food recalls due to microbiological concerns (Ward *et al*. 2004; Ducey *et al*. 2007).

There are 13 known serotypes of *L. monocytogenes* of which the majority occurring in food and associated with human listeriosis belong to serotypes 1/2a, 1/2b and 4b (Miettinen *et al*. 1999; Borucki *et al*. 2004; de Vasconcelos *et al*. 2008; Neves *et al*. 2008). Substantial differences in the ecology and virulence properties of different *L. monocytogenes* serotypes have been documented (de Vasconcelos *et al*. 2008; Ward *et al*. 2010). The *L. monocytogenes* population has been divided into distinct phylogenetic divisions/lineages, namely lineage I, II and III by molecular sub-typing procedures (Roberts *et al*. 2006; den Bakker *et al*. 2008). *L. monocytogenes* serotypes that belong to lineage group I has been linked most often to human listeriosis (Jinneman and Hill 2001; den Bakker *et al*. 2008), suggesting that these strains have an increased virulence or a better adaptation to the human host (Mereghetti *et al*. 2002). Within lineage I, most human listeriosis cases have been caused by serotype 4b (Lin *et al*. 2006; de Vasconcelos *et al*. 2008). Isolates belonging to lineage III are uncommon and have distinctive genetic and phenotypic characteristics (Roberts *et al*. 2006). Various molecular sub-typing

techniques are required to differentiate between *L. monocytogenes* serotypes involved in sporadic and epidemic listeriosis cases for epidemiological investigations (Borucki *et al*. 2004; Roberts *et al*. 2006). A number of rapid and discriminatory sub-typing techniques that have been developed for such investigations, include DNA-based methods such as PFGE, PCR and RFLP analysis and have been critical for epidemiological investigations of *L. monocytogenes* since they contribute greatly to the prevention and the control of human listeriosis (Yde and Genicot 2004; Mammina *et al*. 2009). Pulsed field gel electrophoresis (PFGE) is currently the 'gold standard' of the sub-typing techniques and has proven to be useful in outbreak detection and epidemiological investigations (Chen *et al*. 2005; Harbottle *et al*. 2006; Ward *et al*. 2010).



The occurrence of food-borne disease remains a significant health issue for both developed and developing countries even though governments throughout the world play their part in ensuring food safety. The burden of food-borne disease is generally documented better in developed countries compared to developing countries where there is a demand for adequate health care systems (WHO/INFOSAN 2006).

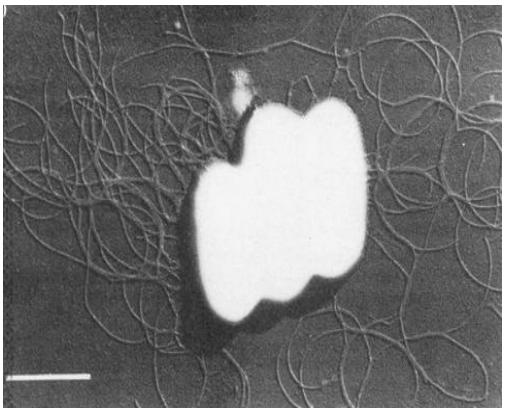
a 

**Figure 2.1a** Transmission electron micrograph of cells of *L. monocytogenes* grown at 20˚C shadowed with gold/palladium metal. An abundant arrangement of flagella around the cell is observed. Bars 0.75µm. Adapted by Peel *et al*. 1988

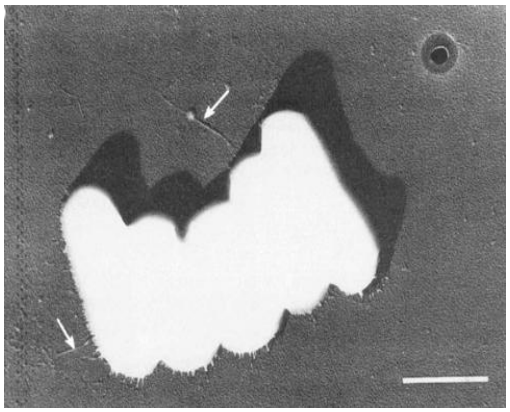
b 

**Figure 2.1b** Transmission electron micrograph of cells of *L. monocytogenes* grown at 37˚C with no fully formed flagella. Arrows denote fragments of flagella only. Bars 0.75µm. Adapted by Peel *et al*. 1988

## 2.2 LEGISLATION AND SURVEILLANCE

The ubiquitous nature and the ability of *L. monocytogenes* to grow at refrigeration temperatures is a challenge to the food industry. Some countries have set legal limits pertaining to the number of organisms, or *L. monocytogenes* that is allowed in food products, especially RTE food products. However some countries, where no legal limits have been set, have provided guidelines or criteria for the number of *L. monocytogenes* allowed in food products (Nogva *et al*. 2000). The United States Food and Drug Association (USFDA) have issued a zero-tolerance ruling for the presence of *L. monocytogenes* 25 $g^{-1}$ in RTE foods namely, products that may be eaten without further cooking or heating (Norton *et al*. 2001; FDA/CFSAN *et al*. 2003; Borucki *et al*. 2004). Since high levels of *Listeria* are difficult to eradicate in food processing environments, the International Commission on Microbiological Specifications for food specified that 100 cfu $g^{-1}$ of *L. monocytogenes* in food was allowed at the time of consumption for non-risk consumers (Rodríguez-Lázaro *et al*. 2004). When a food product is found to be contaminated, the implicated food product is re-called. The United States Department of Agriculture and Food Safety and Inspection Service (USDA/FSIS) has also issued a zero-tolerance ruling for the presence of *L. monocytogenes* 25 $g^{-1}$ in RTE food products whereas some countries, including partners in the United States such as Canada and Denmark, have a non zero-tolerance for *L. monocytogenes* pertaining to certain foods. In Canada, RTE foods that have not been linked to an outbreak and show no *L. monocytogenes* growth within a 10-day period of refrigeration storage may contain up to

100 cfu $g^{-1}$ but cannot exceed 100 cfu $g^{-1}$. The Canadian policy for *L. monocytogenes* in RTE foods is based on the principles of HACCP (FDA/CFSAN *et al*. 2003; Zhu *et al*. 2005).

The annual incidence of listeriosis is estimated to be four cases per million population in Canada (Conly and Johnston 2008). In Toronto, Canada, a listeriosis outbreak in 2008 had occurred and was linked to deli meats (Conly and Johnston 2008). It resulted in approximately 53 cases and 22 deaths (Powell 2009). *L. monocytogenes* on the slicing machine contaminated the packaged cold cuts. The deadly outbreak was referred to as a preventable incident and the Canadian Prime Minister appointed an individual (the former head of the health system) who constructed a report which addressed four broad categories where improvements in food safety had to be made (Powell 2009). The report also stated that both in the private and public sector, the tragedy was a result of a lack of attention to food safety by senior management (Galloway 2009). The company, Maple Leaf Foods, paid 27 million dollars to the victims of families of the outbreak (Galloway 2009).



With regard to Australia's National Food Standard Council, regulations require that pâté, soft cheeses, smoked fish and smoked sea-food have zero *Listeria* at the point of wholesale distribution. Also, the control of *Listeria* in the dairy industry sets out procedures to prevent *Listeria* contamination in the processing plant; namely, milk and milk products and thereby clear a product intended for sale should *Listeria* be found in

the dairy product or the environment of the processing plant (Victorian Government Health Information 2004). A recent listeriosis outbreak in 2009 in Australia, linked to contaminated airline food, resulted in two pregnant women giving birth prematurely. Both women and their babies survived. The source of contamination was chicken wraps sold in their thousands on the airline and as a result it triggered a national public health alert and the chicken wraps were withdrawn from the airline (Walker 2009).

The Food Standards Australia New Zealand (FSANZ) issued a zero-tolerance ruling for cooked crustacea and processed molluscs and allows < 100 cfu $g^{-1}$ in one out of five samples of RTE processed finfish (Bremer *et al*. 2003). The potential for the number of listeriosis cases in New Zealand to increase exists since the number of the elderly population is increasing as is the availability of chilled RTE foods with an increased shelf life (Crerar *et al*. 2010). The New Zealand Food Safety Authority (NZFSA) issued a statement of intent for 2008-2011 which stipulated that "we will improve the safety of food to reduce the incidence of food-borne illness". This statement also proposed a performance target for *L. monocytogenes* of "no increase in the reported incidence of food-borne illness after five years" (Crerar *et al*. 2010).



Extensive work has been done in Europe to reduce the incidence of listeriosis (Lundén *et al*. 2004). According to the European Community Directive for milk and milk-based products, a zero-tolerance ruling for the presence of *L. monocytogenes* in soft cheeses has been issued and the organism must be absent in 1 g of other products (Nogva *et al*. 2000).

The European Commission has issued a ruling for a maximum level of 100 cfu $g^{-1}$ in RTE foods at the end of shelf life (Valero *et al*. 2006; Baudouin *et al*. 2010). Most countries in the European Union have an annual incidence of listeriosis of between two and 10 reported cases per million per year (Jemmi and Stephan 2006). Due to a rise in the number of listeriosis cases in Europe in the recent years, there's been a call for an enhanced surveillance system at the European Union level to have a better understanding of the prevalence of the organism in the food environment and the burden of disease (Mammina *et al*. 2009; Pinto 2010).



With regard to South African policy, the Department of Health's legislation specified the following; some foods permit the growth and proliferation of *Listeria* with regard to certain factors e.g. storage temperature. For these foods, the limit for the presence of *L. monocytogenes* should be zero. For foods that do not support the growth of *Listeria* the limit is 100 cfu $g^{-1}$. The Department of Health have different specifications with regard to various food items (Table 2.1) (DOH 2001). Reducing the rates of listeriosis by considering control measures which lower the levels of contamination at consumption may be advantageous. Foods which do not support the growth of *L. monoytogenes* during storage have a lower risk per serving e.g. ice-cream. Where small numbers of *L. monocytogenes* are present in foods during manufacturing and encouraged to grow during prolonged storage due to favourable temperature conditions, these foods are those which permit growth during storage and have a higher risk for causing listeriosis (Makhoane 2003).

**Table 2.1** Specifications for the presence of *L. monocytogenes* in various food products as indicated by the Department of Health, South Africa (DOH 2001).

| ***L. monocytogenes*** | **Food item** | **Limits** |
|---|---|---|
| | Cheese | 0 cfu $g^{-1}$ |
| | Cold meal items:<br>Cold meats, processed meats, polony, dried vegetables, ham, potato salad with mayonnaise.<br>Cold smoked or fermented meal items:<br>Salami, bacon, buns, bread, smoked cold meat, caviar. | < 10 cfu $g^{-1}$ |
| | Items requiring further cooking:<br>Blanched and frozen vegetables, half-cooked meals (also steak, chops, wors), raw meat, meat basting sauce. | < 1000 cfu $g^{-1}$<br>< 100 cfu $g^{-1}$ |
| | Cooked items before cooling:<br>Pastry, bulk vegetables, deep fried potato chips, pizza's, RTE frozen meals, tinned jam, tinned vegetables | 0 cfu 25 $g^{-1}$ |

According to the editor of Food For Thought, Makhoane (2003); in South Africa, food-borne disease in many cases is under-reported, which is a result of there being an absence of an efficient and integrated surveillance and notification system for food-borne disease. The legislation specifies that where four or more cases are observed by the same doctor or health facility, only then should those food poisoning cases be reported. In turn, most cases are never reported due to patients that do not seek medical attention since the food-borne illnesses are self-limiting. Many food poisoning cases and occasionally deaths go unnotified since the illness and causative agent is not identified and confirmed by laboratory tests. Medical practioners are offered no incentives to follow up on these cases. Makhoane (2003) concluded that an efficient food-borne disease surveillance system is required in South Africa.



Quantitative risk assessment of microbiological hazards was the work of Codex Alimentarius Commission (CAC). The Codex Committee on Food Hygiene (CCFH) identified a list of pathogens for which expert advice was required for risk assessment. The International Food Safety Authorities Network (INFOSAN) was a joint initiative between the World Health Organization (WHO) and the Food and Agricultural Organization (FAO) of the United States who provided the expert advice on risk assessments of microbiological hazards in foods. These risk assessments were of value and importance to South Africa since it was used as a guideline for determining microbiological specifications for *L. monocytogenes* in RTE foods sold or imported into South Africa (Makhoane 2003).

South Africa has a routine notification system for reporting medical conditions that are notifiable. Of the medical conditions that are notifiable, food poisoning is regarded as a category A type of disease. This category includes medical conditions that have to be reported immediately by telephone or fax to the regional/provincial or department of health (DOH) when the diagnosis is made. Notification should be immediate regardless of whether the condition is presumptive or confirmed. The GW17/5 (cased-based form) should follow within 5 days (DOH accessed January 08, 2011) (Figure 2.2).

Compared to countries that have developed surveillance systems for listeriosis, it has been reported that there are few or even no reports from Africa, Asia and South America (Rocourt and Cossart 1997; Lemes-Marques *et al*. 2007). No specific national surveillance for listeriosis is conducted in South Africa (NICD 2007). According to the NICD report (2007) which provides up-to-date information on communicable diseases in South Africa, five culture confirmed cases of human listeriosis by *L. monocytogenes* were identified in the Eastern Cape region between December and January 2007. Symptoms of meningitis were displayed and all patients died within one to three days of hospitalization. The age of the patients ranged from 26 days to 46 years, two of which were HIV-positive and the others had compromised immune systems. In the KwaZulu Natal region, four cases were reported between January and February 2007 of which the clinical outcomes were not confirmed. Cases included a three month old infant, two adults in their twenties with a compromised immune system and an elderly diabetic. A

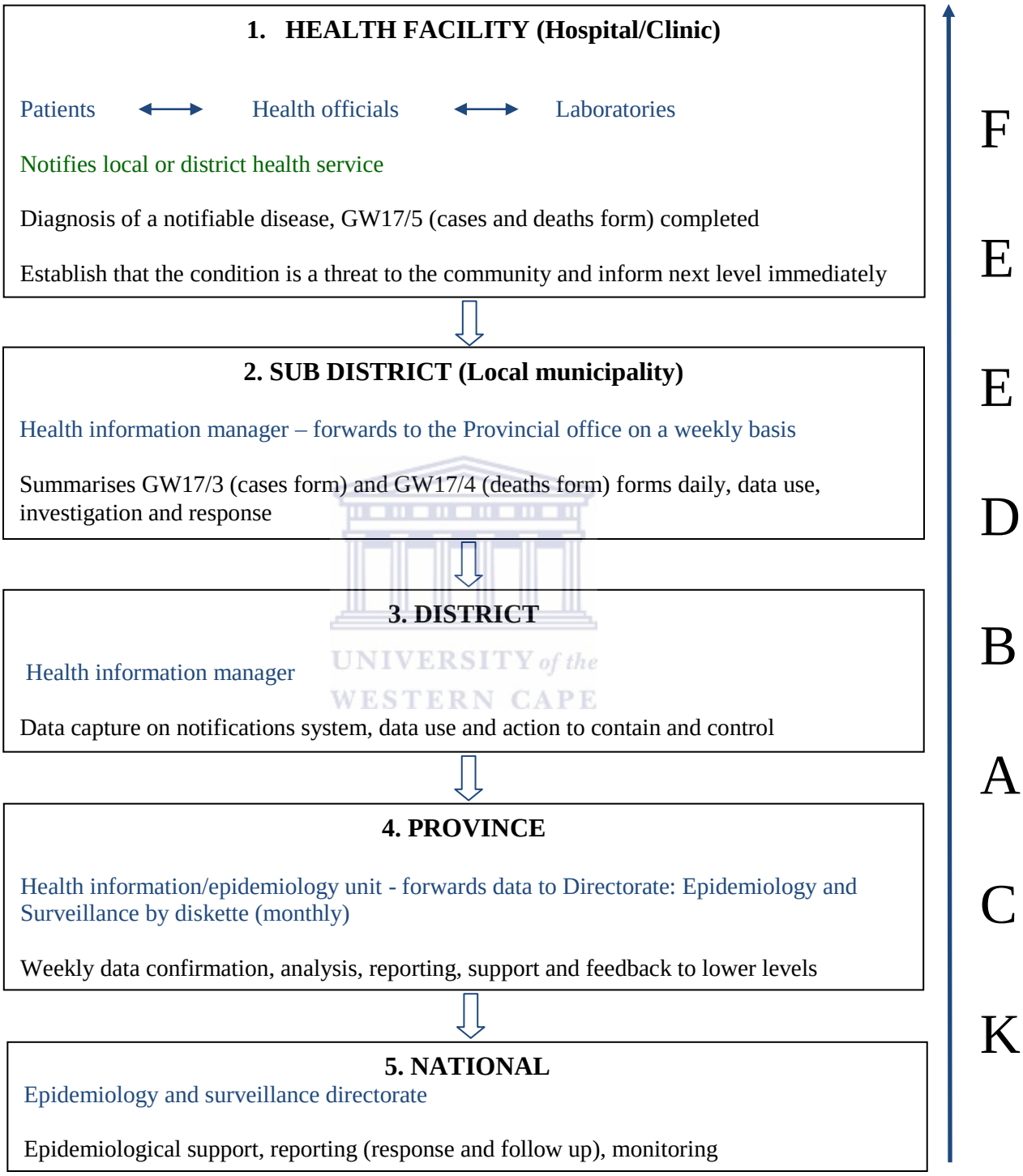



**Figure 2.2** Schematic representation of information for notifying a medical condition in South Africa (adapted from DOH, accessed January 08, 2011).

shortage of resources for adequate investigations of disease outbreaks leads to the difficulty in controlling *L. monocytogenes* illness.

The WHO has developed five keys to safer foods in order to prevent food-borne disease (WHO/INFOSAN 2006). These guidelines were developed in order to reduce the millions of people that became ill daily and the thousands that died from food-borne disease. The five keys to safer food include; keep clean, separate raw and cooked, cook food thoroughly, keep food at safe temperatures and use clean safe water and raw materials (WHO/INFOSAN 2006). These guidelines were translated into 40 languages in order for it to be understood by diverse groups.



The United States Department of Health and Human Services (US/DHHS) co-coordinated a 'Healthy people 2010' project worked on by the FDA, FSIS and Centre for Disease Control (CDC) in order to achieve an additional 50% reduction in listeriosis by the year 2010 in the USA. This initiative aimed to prevent disease and promote health by bringing together various organizations, government and communities to improve the quality of life (FDA/CFSAN *et al*. 2003). This objective has moved towards its target; between 1997 and 2002, *L. monocytogenes* infections declined from 0.47 cases per 100 000 population to 0.26 cases per 100 000 population achieving 91 percent of the targeted change i.e. 0.24 cases per 100 000 population for 2010. From 2005-2008, the *L. monocytogenes* infections were 0.3 cases per 100 000 population (CDC: Data 2010). The reduction/absence of *L. monocytogenes* in food products will prevent economic

consequences like the withdrawal of products leading to a decrease in sales for products suspected of being contaminated. Usually the presence of any *Listeria* species in food is an indicator of poor hygiene (Cocolin *et al*. 2002; Ford 2010). To reduce the burden of food-borne disease requires an increase and improved effort and collaboration by the government, scientists, food industry and consumers.

## 2.3 FOOD-BORNE LISTERIOSIS

Listeriosis is acquired by eating food that is contaminated with *Listeria* (Zhou and Jiao 2005). Although the incidence of infection is low, listeriosis has a high mortality rate especially within vulnerable groups namely the elderly, infants and the immuno-compromised (Elliot and Kvenberg 2000; Lundén *et al*. 2004; Besse *et al*. 2005; Nappi *et al*. 2005).



The occurrence of listeriosis is quite low with an annual incidence of two to 15 reported cases per million in the USA and two to 10 reported cases per million in the European Union countries, although the mortality rate is about 20-30% and a 90% hospitalization rate for those with listeriosis (Kwiatek 2004; Zhang *et al*. 2004a; Dawson *et al*. 2006; Hain *et al*. 2006) (Table 2.2 and Table 2.3). This is high compared to a 0.04% death rate with food-borne salmonellosis (Doyle 2001) which is responsible for more cases of food-borne illness (Tables 2.4 - 2.6). The mortality rate of infection with *L. monocytogenes* far exceeds that of other common food-borne pathogens (Liu 2006). The minimal infective

dose is estimated to be > 100 cfu $g^{-1}$ with listeriosis cases being sporadic but occasionally also epidemic (DOH 2001; Holko *et al*. 2002), however incidences of infection related to fewer than 100 cfu $g^{-1}$ in food were reported (Willis *et al*. 2006). An example involved an outbreak linked to frankfurters containing less than 0.3 cfu $g^{-1}$ *L. monocytogenes*, suggesting that the strain responsible had increased virulence.

The Foodborne Disease Active Surveillance Network (FoodNet) has a surveillance system in 10 states of America for laboratory-confirmed cases of infection caused by the pathogens listed in Table 2.2 and Table 2.3 (CDC: FoodNet 2010).



**Table 2.2** Preliminary data for the number of lab confirmed bacterial infections in the USA from 1996-2009 (adapted from CDC: FoodNet 2010)

| **Pathogen** | **1996** | **1997** | **1998** | **1999** | **2000** | **2001** | **2002** | **2003** | **2004** | **2005** | **2006** | **2007** | **2008** | **2009** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Campylobacter* | 3367 | 3960 | 4022 | 3832 | 4710 | 4751 | 5064 | 5272 | 5686 | 5692 | 5771 | 5872 | 5857 | 6033 |
| *Listeria* | 65 | 76 | 113 | 118 | 105 | 94 | 98 | 139 | 119 | 136 | 139 | 122 | 135 | 158 |
| *Salmonella* | 2064 | 2186 | 2820 | 4155 | 4315 | 5240 | 6149 | 6038 | 6498 | 6506 | 6690 | 6829 | 7458 | 7039 |
| *Shigella* | 1269 | 1269 | 1480 | 966 | 2350 | 2219 | 4113 | 3039 | 2248 | 2095 | 2765 | 2869 | 3044 | 1849 |
| STEC 0157* | 374 | 337 | 491 | 502 | 623 | 542 | 641 | 444 | 402 | 473 | 590 | 547 | 518 | 459 |
| STEC non 0157 | -† | - | - | - | 36 | 61 | 35 | 47 | 110 | 128 | 227 | 270 | 228 | 264 |
| *Vibrio* | 21 | 52 | 50 | 48 | 54 | 79 | 104 | 110 | 123 | 121 | 156 | 110 | 136 | 160 |
| *Yersinia* | 147 | 139 | 181 | 162 | 133 | 144 | 169 | 162 | 176 | 163 | 163 | 164 | 166 | 150 |

* Shiga toxin-producing *Escherichia coli*

† Surveillance not conducted in this year

**Table 2.3** The number of laboratory confirmed bacterial infections in 2009 in the USA according to pathogen and age group* (adapted from CDC: FoodNet 2010)

| | Age group (years) | | | | | |
|---|---|---|---|---|---|---|
| **Pathogen** | **<4** | **4-11** | **12-19** | **20-49** | **≥50** | **Total** |
| *Campylobacter* | 717 | 549 | 424 | 2455 | 1879 | 6024 |
| *Listeria* | 17 | 0 | 4 | 23 | 114 | 158 |
| *Salmonella* | 1822 | 943 | 515 | 1986 | 1764 | 7030 |
| *Shigella* | 415 | 594 | 109 | 541 | 187 | 1846 |
| STEC 0157 | 97 | 103 | 85 | 105 | 69 | 459 |
| STEC non 0157 | 68 | 40 | 39 | 79 | 38 | 264 |
| *Vibrio* | 2 | 4 | 7 | 61 | 86 | 160 |
| *Yersinia* | 59 | 13 | 6 | 35 | 37 | 150 |

*Data are preliminary. There were 24 records with missing age which were not included



The percentage of hospitalization is determined by dividing the number of patients who were hospitalized (only where it occurs within seven days of the specimen collection date) by the number of cases reported (Table 2.4 and Table 2.5).

**Table 2.4** Preliminary data for the number and percentage (%) of hospitalizations in 2009 in the USA (adapted from CDC: FoodNet 2010)

| Pathogen | Total number (no) | | |
|---|---|---|---|
| | **No. hospitalization** | **cases** | **% hospitalization** |
| *Campylobacter* | 877 | 6033 | 14.5 |
| *Listeria* | 141 | 158 | 89.2 |
| *Salmonella* | 1936 | 7039 | 27.5 |
| *Shigella* | 370 | 1849 | 20.0 |
| STEC 0157 | 192 | 459 | 41.8 |
| STEC non 0157 | 39 | 264 | 14.8 |
| *Vibrio* | 48 | 160 | 30.0 |
| *Yersinia* | 40 | 150 | 26.7 |

**Table 2.5** Preliminary data* including the number and % of hospitalizations in 2009 represented by age group in the USA (adapted from CDC: FoodNet 2010)



| | **Age group (years)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **<4** | | | **4-11** | | | **12-19** | | | **20-49** | | | **≥50** | | |
| | **Total no** | | | **Total no** | | | **Total no** | | | **Total no** | | | **Total no** | | |
| | **No. hosp** | **Cases** | **% hosp** | **No. hosp** | **cases** | **% hosp** | **No. hosp** | **Cases** | **% hosp** | **No. hosp** | **Cases** | **% hosp** | **No. hosp** | **cases** | **% hosp** |
| *Campylobacter* | 71 | 717 | 9.9 | 57 | 549 | 10.4 | 59 | 424 | 13.9 | 289 | 2455 | 11.8 | 400 | 1879 | 21.3 |
| *Listeria* | 16 | 17 | 94.1 | 0 | 0 | NA | 3 | 4 | 75.0 | 20 | 23 | 87.0 | 102 | 114 | 89.5 |
| *Salmonella* | 406 | 1822 | 22.3 | 193 | 943 | 20.5 | 93 | 515 | 18.1 | 446 | 1986 | 22.5 | 797 | 1764 | 45.2 |
| *Shigella* | 50 | 415 | 12.0 | 94 | 594 | 15.8 | 28 | 109 | 25.7 | 143 | 541 | 26.4 | 55 | 187 | 29.4 |
| STEC 0157 | 30 | 97 | 30.9 | 41 | 103 | 39.8 | 41 | 85 | 48.2 | 39 | 105 | 37.1 | 41 | 69 | 59.4 |
| STEC non 0157 | 3 | 68 | 4.4 | 1 | 40 | 2.5 | 5 | 39 | 12.8 | 17 | 79 | 21.5 | 13 | 38 | 34.2 |
| *Vibrio* | 0 | 2 | 0.0 | 0 | 4 | 0.0 | 1 | 7 | 14.3 | 12 | 61 | 19.7 | 35 | 86 | 40.7 |
| *Yersinia* | 14 | 59 | 23.7 | 3 | 13 | 23.1 | 1 | 6 | 16.7 | 6 | 35 | 17.1 | 16 | 37 | 43.2 |

hosp = hospitalization

*information for 24 records with missing age not included

The percentage for the case fatality rate is determined by establishing the number of case-patients reported to FoodNet who died divided by the number of cases reported to FoodNet. Monitoring in the USA, show that there's a greater likelihood of being hospitalized by illness caused by *Listeria* than any other food-borne pathogen (Curtis 2007) (Table 2.6).

**Table 2.6** Preliminary data for the number of deaths and case fatality rates caused by pathogens in the USA during the year 2009 (adapted from CDC: FoodNet 2010)

| Pathogen | Number of deaths | Total number of cases | Case fatality rate |
|---|---|---|---|
| *Campylobacter* | 9 | 6033 | 0.15 |
| *Listeria* | 20 | 158 | 12.66 |
| *Salmonella* | 24 | 7039 | 0.34 |
| *Shigella* | 1 | 1849 | 0.05 |
| STEC 0157 | 2 | 459 | 0.44 |
| STEC non 0157 | 1 | 264 | 0.38 |
| *Vibrio* | 7 | 160 | 4.38 |
| *Yersinia* | 2 | 150 | 1.33 |



The host's immune defense system and the strain of *L. monocytogenes* involved in the illness determine the host's response to disease (Vasilev *et al*. 2010). *L. monocytogenes* is present in food usually at low numbers (< 10 cfu $g^{-1}$) however it has been found in larger numbers (> 10 000 cfu $g^{-1}$) in foods such as pâtés and soft cheeses (Curtis 2007). Listeriosis has always been regarded as an invasive disease affecting susceptible groups, but a non-invasive form of listeriosis that causes febrile gastroenteritis, headaches, nausea and vomiting in healthy adults has increased public awareness of *L. monocytogenes* due

to the expanding vehicle of infection (Franciosa *et al*. 2001; Lundén *et al*. 2004; Nappi *et al*. 2005).

As previously mentioned; among the *Listeria* spp. only *L. monocytogenes* is known to cause disease. However, there has been an incidence of where a clinical case of bacteremia was documented which resulted in the death of the patient. The organism responsible was *L. innocua*. According to the authors' knowledge, this was the first description of a human infection caused by *L. innocua* (Perrin *et al*. 2003). Another human clinical case of bacteremia was reported; however this time the organism responsible was *L. ivanovii*. The patient survived after receiving medical treatment. Generally, the occurrence of infections in humans by *Listeria* spp other than *L. monocytogenes* is rare. Since *L. ivanovii* is only isolated occasionally from environmental or animal sources, it may indicate a limited distribution in nature (Guillet *et al*. 2010). *L. ivanovii* can invade mammalian cells but unlike *L. monocytogenes*, it lacks the cytotoxin which is suggestive of the reduced virulence displayed by *L. ivanovii* (Koneman *et al*. 2006).

## 2.4 GROWTH OF *LISTERIA* IN FOODS; INCIDENCE AND OUTBREAKS

*Listeria monocytogenes* and *L. ivanovii*, species of the genus *Listeria* are both commonly found in rotting vegetation, soil and water. Only the species *L. monocytogenes* has been recognized as a human pathogen responsible for several epidemics of listeriosis. Several sources have been identified as possible routes for *L. monocytogenes* transmission to humans (Figure 2.3) (Axelsson and Sorin 1998). Since *Listeria* can survive and grow under adverse conditions including a low pH, low refrigeration temperature and high salt concentration (Table 2.7), they easily contaminate food and become a concern to the food industry (Lundén *et al*. 2004; Burnett *et al*. 2005). *L. monocytogenes* has the ability to be present in all raw foods while its presence in cooked food may be attributed to post-process contamination (Curtis 2007). The fact that *L. monocytogenes* has been implicated in outbreaks and sporadic cases due to their prevalence in dairy and meat products has resulted in serious economic losses due to product recalls (Besse *et al*. 2004; Leite *et al*. 2006).



The ability of *L. monocytogenes* to form biofilms after attaching to a wide variety of food contact surfaces presents reasons for concern when cleaning or disinfection procedures are applied. A biofilm is formed when a community of *L. monocytogenes* attaches to a surface in an extracellular matrix. Its complete eradication from a food-processing plant or abattoir is almost unachievable. Aside from other locations, biofilms can occur on

floors, freezers, waste water pipes, bends in pipes and processing rooms (Dabrowski *et al*. 2003; Dharmarha 2008).

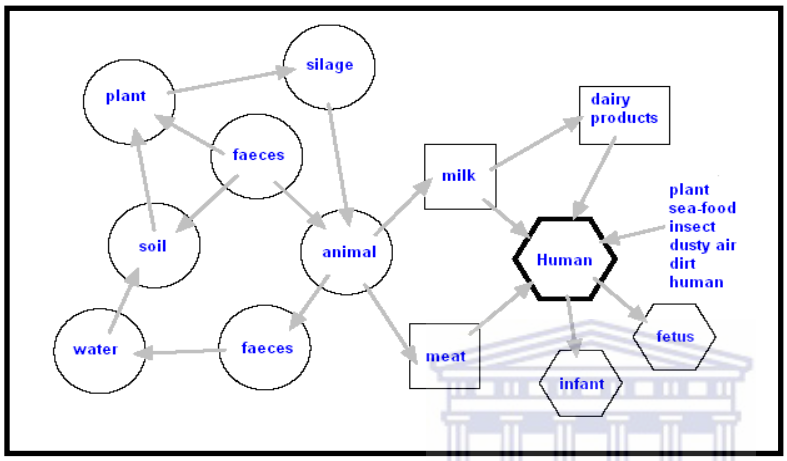



**Figure 2.3** Implicated routes of transmission for *L. monocytogenes* infection to humans (adapted from Axelsson and Sorin 1998).



**Table 2.7** Conditions for pathogen growth (adapted from Bremer *et al*. 2003; Curtis 2007)

| | **Conditions for Pathogen Growth** | | | | | | |
|---|---|---|---|---|---|---|---|
| **pathogen** | **min. $a_w$** | **min. pH** | **max. pH** | **max. % salt** | **min. temp.** | **max. temp.** | **oxygen requirements** |
| *Listeria monocytogenes* | 0.92 | 4.3 | 9.6 | 10 | 0-2°C | 45°C | facultative anaerobe |

### 2.4.1 Contamination of dairy products

Dairy products have been implicated in the transmission of human listeriosis (Kérouanton *et al*. 1998) of which raw milk has been implicated (Lundén *et al*. 2004). The ability of *Listeria monocytogenes* to contaminate dairy food is a lethal threat to the dairy industry. Dairy products such as raw milk samples and soft cheese have shown to be vehicles of contamination during *L. monocytogenes* outbreaks and the number of outbreaks associated with dairy products accounts for half the number of outbreaks caused by all food types (Cox *et al*. 1998).



Milk and milk products are highly perishable foods that may be potentially unsafe to consume due to the growth of micro-organisms (Xanthiakos *et al*. 2006). Raw/unpasteurized milk or food made from raw milk may contain *L. monocytogenes* (Schett *et al*. 2005). *Listeria* are killed by pasteurization, however contamination may occur after pasteurization but before packaging (CDC 2005). An example is butter made from pasteurized milk, which proved to be contaminated in subsequent stages of production (Lundén *et al*. 2004). An increase in the number of listeriosis cases in Northern England was linked to the consumption of contaminated butter (Greenwood *et al*. 2005).

Soft cheeses manufactured from pasteurized milk have also been linked to listeriosis outbreaks suggesting that raw milk is not the only risk product (Lundén *et al*. 2004). The

first outbreak of listeriosis that resulted in the recall of a food product was linked to soft cheeses and the outcome was the shutdown of the food processing plant (Conly and Johnston 2008). Although the pasteurization of raw milk is considered as being efficient to control/prevent *L. monocytogenes* contamination, chances are that the product may become contaminated after this heat treatment. An outbreak in 1994 in Illinois caused 45 people to become ill. The outbreak was linked to chocolate milk that contained *L. monocytogenes* serotype 1/2 b (Doyle 2001). Factors that influence milk composition and microbial load include hygienic practices of farmers namely, the washing of milking equipment and udder preparation for milking. Where the milk is stored at a low temperature, *Pseudomonas* spp. together with *Listeria* may proliferate. Where the udder is not cleaned efficiently, salt tolerant micro-organisms tend to proliferate (Lafarge *et al*. 2004). Where milk and milk products are not properly processed, packaged, distributed and stored, micro-organisms may proliferate and make the product unsafe for consumption (Xanthiakos *et al*. 2006).



One of the food products most frequently associated with listeriosis are soft cheeses, especially those made from unpasteurised milk (Holko *et al*. 2002). A heat treatment that is given during cheese manufacturing is usually sufficient in killing *L. monocytogenes* that may be present (Longhi *et al*. 2003), although post-processing contamination is a possibility (Longhi *et al*. 2003; Pinto *et al*. 2010). *L. monocytogenes* tolerates harsh conditions and therefore can grow and survive in different types of food. *L. monocytogenes* tolerates conditions such as a low $a_w$ and a high salt concentration, which

facilitates its survival in products with a high fat content, example semi-hard cheese (Lundén *et al*. 2004). The growth and survival of *L. monocytogenes* in soft cheeses is favoured by their maturation and storage at refrigeration temperatures (Leite *et al*. 2006). The prevalence of *L. monocytogenes* in different types of cheeses varies. In soft and semi-soft cheeses the $a_w$ is higher than in hard cheeses, facilitating faster growth for the organism whereas in mold cheese, during the ripening process, pH levels approach neutral facilitating the growth of *L. monocytogenes* (Lundén *et al*. 2004). During ripening, an increase in the pH of cheese is paralleled by an increase in the growth of the organism, which results in contamination being centered on the surface of the rind (Pearson and Marth 1990; Farber and Peterkin 1991). *L. monocytogenes* can survive a number of cheese-making processes (manufacturing and ripening) if present in raw milk (100 cfu $ml^{-1}$) and remain viable in the final product for a long time. It survives best in cheeses such as camembert and worst in cottage cheese. *L. monocytogenes* tends to be concentrated in the curd and scarcer in the whey (Griffiths 1989; Pearson and Marth 1990; Farber and Peterkin 1991).



During the 1980's, the incidence of reported *Listeria* infections increased drastically together with the number of food related outbreaks. In 1985, in Los Angeles, a listeriosis outbreak attributed to Mexican style cheese was responsible for 142 cases and 48 deaths while during the late 1980's, in the UK, a listeriosis outbreak was associated with pâté responsible for > 350 cases and > 90 deaths (Curtis 2007).

Healthy cows can serve as reservoirs for *L. monocytogenes* whereby the organism is secreted in its milk. Milk can also become contaminated through accidental contact with feces and silage (Axelsson and Sorin 1998).

### 2.4.2 Contamination of meat and poultry

*Listeria monocytogenes* has been associated with a variety of foods including RTE foods and is a well-known problem in production environments including abattoirs and meat processing plants (Purwati *et al*. 2001). Studies showed that *L. monocytogenes* strains isolated from meat or RTE food resulted from the processing environment rather than the animal itself (Harvey and Gilmour 1994; Beumer and Hazeleger 2003).



Meat and meat products have frequently been contaminated with *L. monocytogenes* where the organism has shown to proliferate through frozen foods (Mahmood *et al*. 2003) and poses a risk to the safety of RTE meat products (Figure 2.4) (Purwati *et al*. 2001; Cocolin *et al*. 2004; Rodríguez-Lázaro *et al*. 2004; Zhu *et al*. 2005). *L. monocytogenes* began to emerge as a problem associated with processed meat and poultry during the 1980's (FSIS 2003) and since has been commonly isolated from different types of processed meats where it has shown to have an increase in growth in high pH cooked meat and poultry products (Samelis and Metaxopoulos 1999). It has been shown that *L. monocytogenes* grows fairly well in meats and poultry products with a pH near or above 6.0 and poorly or not at all below pH 5.0 (Zhu *et al*. 2005). Low pH cured meats e.g.

fermented sausages do not pose much risk to the consumer since only a few *L. monocytogenes* cells may survive in the product. The use of poultry meat to produce processed meats has paralleled an increase in the level of *Listeria* in meat products as chicken, turkey breasts, wings and drumsticks have shown a high incidence of haemolytic and non-haemolytic *Listeria* spp. (Samelis and Metaxopoulos 1999).

Frankfurters has been a source of *L. monocytogenes* food poisoning when a cancer patient died developing meningitis (Mahmood *et al*. 2003). Luncheon meats and hotdogs were implicated in a listeriosis outbreak in the United States in the late 1990's, which resulted in 101 illnesses and 21 deaths - 15 adult deaths and 6 miscarriages (FSIS 2003). An outbreak of listeriosis was linked to the consumption of pork tongue in France in 1999/2000 (Greenwood *et al*. 2005).



RTE cooked meats are commonly contaminated with *L. monocytogenes* during slicing and packaging after cooking or during the post-processing steps which is a concern since RTE cooked meats that do not require further cooking have a long shelf life and *L. monocytogenes* also has the ability to grow at refrigerated temperatures in the presence of a high salt concentration used for preserving meat products (Mahmood *et al*. 2003; Kwiatek 2004; Bruhn *et al*. 2005; Zhu *et al*. 2005). In a study undertaken by Samelis and Metaxopoulos (1999), *Listeria* were not detected in sausages heated in their final packs neither in the fully ripened dry salamis suggesting that contamination was likely to occur after cooking or during post-processing steps. Fresh sausages are very perishable

products with a $a_w$ equal to or higher than 0.97 and a pH value not lower than 5.5 (Cocolin *et al*. 2004).

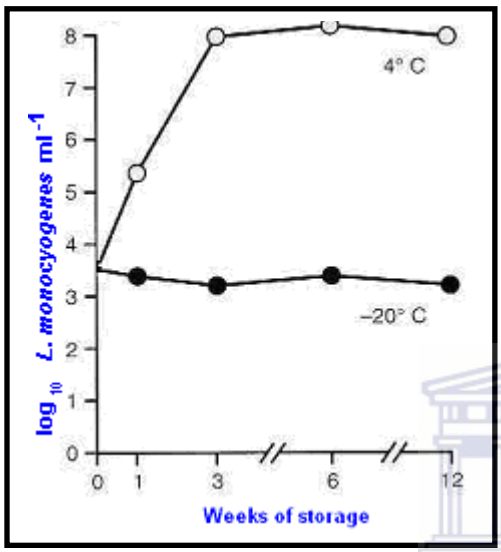





**Figure 2.4** Growth and survival of *L. monocytogenes* in certain foods at 4˚C and -20˚C. (Adapted from Todar 2008).

### 2.4.3 Contamination of seafood

The consumption of seafood contaminated with *Listeria monocytogenes* has been implicated with human listeriosis (Agersborg *et al*. 1997; Kwiatek 2004; Conly and Johnston 2008). *L. monocytogenes* has been isolated from fresh, frozen and processed products including crustaceans, molluscan shellfish and finfish (Elliot and Kvenberg 2000; Bremer *et al*. 2003). A time / temperature guidance for controlling pathogen growth and toxin formation is seen in Table 2.8.

Cold smoked fish products and hot smoked mussels are RTE food products usually eaten without cooking and have posed a risk for contamination with *L. monocytogenes* since there is a lack of a heat inactivation step during processing (Boerlin *et al.* 1997; Norton *et al*. 2001; Bremer *et al*. 2003; Besse *et al*. 2004). The salt content, pH and $a_w$ levels usually facilitate the growth and survival of *L. monocytogenes* (Rørvik 2000). Cold smoked salmon is a RTE food product that is smoked to add flavour as well as to preserve the product by preventing the growth of micro-organisms. Since this procedure is performed at a temperature below 28˚C, it is not always effective in eliminating micro-organisms that may have been on the raw product prior to smoking (Simon *et al*. 1996). The smoking of salmon could involve cold smoking, which tends to reduce the level of *L. monocytogenes* but not eliminate them since the temperature is too low, or hot smoking (where smoke is applied during the whole heating process) at a temperature higher than 60˚C to eliminate the micro-organisms (Rørvik 2000).



In a study undertaken by Boerlin *et al*. (1997) to determine whether specific strains of *L. monocytogenes* were specific for particular types of fish products, it was found that there was no association between a specific population of *L. monocytogenes* and sea-food products, however it was found that most of the *L. monocytogenes* isolated from smoked salmon belonged to serotype 1/2 (Rørvik 2000). Subsequent research showed that serotype 1/2a is most frequently isolated from seafood products (Chou and Wang 2006).

A popular sea-food dish in Sweden, 'gravad' rainbow trout, is prepared by mixing rainbow trout with sugar, salt, pepper and dill, where it is placed in a plastic bag and refrigerated for 2 days. Thereafter, it is vacumn packed and stored at 8ºC for up to 21 days. *L. monocytogenes* has been implicated in the contamination of such fish products where it can proliferate at the refrigeration temperature and develop into high numbers (Ericsson and Stålhandske 1997). *L. monocytogenes* has shown not only to survive, but increase in numbers when present on vacumn packed smoked sea-food products that is stored at 4° or 10°C. Where shrimp, crabmeat and other sea-food products were artificially contaminated with *L. monocytogenes* and stored at 7ºC, their numbers increased by 100 000 bacteria $g^{-1}$ within 14 days (Bremer *et al*. 2003).



An outbreak of listeriosis in Sweden in the 1990's was linked to the contamination of cold-smoked rainbow trout and 'gravad' trout which caused 2 deaths. A correlation between the ingestion of cold smoked fish and human listeriosis was found since the same *L. monocytogenes* strains were recovered from the fish products and the patients (Besse *et al*. 2004).

Where *L. monocytogenes* contamination has occurred, the cause has commonly been traced to harvesting, handling, processing or sanitation namely, the production environment (Beumer and Hazeleger 2003; Bremer *et al*. 2003). Contaminated processing surfaces have been believed to be the biggest source of *L. monocytogenes* contamination of sea-food. A study undertaken by Autio *et al*. (1999) showed that *L. monocytogenes*

contamination of fish occurred during processing (brining and slicing) contrary to belief that *L. monocytogenes* contamination of cold smoked rainbow trout was a result of the raw fish. Various studies concluded that raw fish is not a major source of contamination; however slaughtered fish from slaughterhouses may introduce the bacteria and contaminate the plant (Rørvik 2000).

**Table 2.8** Time/Temperature guidance for controlling *L. monocytogenes* growth and toxin formation in seafood (USFDA/CFSAN 2001)

| **Time/Temperature Guidance for Controlling Pathogen Growth and Toxin Formation in Seafoods** | | |
|---|---|---|
| **Pathogen** | **Product Temperature** | **Maximum Exposure Time** |
| Growth of *Listeria monocytogenes* | -0.4-5°C | 7 days |
| | 6-10°C | 2 days |
| | 11-21°C | 12 h |
| | above 21°C | 3 h |



### 2.4.4 Contamination of vegetables

*Listeria monocytogenes* occurs on fresh vegetables and can grow and survive on fresh produce stored at refrigeration temperature (Thomas *et al*. 1999; González-Fandos *et al*. 2001). Vegetables can become contaminated from the soil or manure, which is used as fertilizer and has the ability to grow and survive under conditions associated with processing and storage of raw fruit and vegetables (Li *et al*. 2002; CDC 2005).

The first outbreak of listeriosis linked to a food source was in 1981 (Gasanov *et al*. 2005; Curtis 2007) and caused by commercially prepared coleslaw (Li *et al*. 2002; Conly and Johnston 2008). The epidemic strain was isolated from coleslaw obtained from a patient's refrigerator (Conly and Johnston 2008). This occurred in Canada and 41 cases of listeriosis were presented with 18 deaths resulting. The source of contamination was from fertilizer which was prepared from manure infected with *Listeria* from sheep. The fertilizer was used when growing the cabbages to be used for the coleslaw (Curtis 2007). *Listeria* may be present in high numbers on the surface of vegetables where cell tissue has lost its strength and decay is more prevalent (Beumer and Hazeleger 2003). *L. monocytogenes* has been associated with the consumption of unwashed raw vegetables and cabbage (Mahmood *et al*. 2003) and lettuce, celery and tomatoes were implicated in an outbreak in eight Boston hospitals (Li *et al*. 2002). In New Zealand in 2001, a listeriosis outbreak was linked to unwashed vegetables, salad that had been cross contaminated with raw poultry and some other food products (Bremer *et al*. 2003).

## 2.5 SUB-TYPING

Sub-typing procedures are very important to track individual strains of *L. monocytogenes* that are involved in listeriosis outbreaks as well as to determine the population genetics and epidemiology of this organism (Ragon *et al*. 2008; Jeyaletchumi *et al*. 2010). Sub-typing procedures include phenotypic as well genetic sub-typing methods.

### 2.5.1. Phenotypic sub-typing methods



#### 2.5.1.1 Serotyping

Serological typing is based on antibodies that react with whichever antigens of *Listeria* species are displayed since various strains carry different antigens on their surface (Wiedmann 2002; Gasanov *et al*. 2005). *Listeria* species can be sub-divided into serotypes by means of their antigenic variation namely heat-stable somatic (O) and heat-labile flagellar (H) antigens. *L. monocytogenes* has 13 serotypes, some of which are common to *L. innocua* and *L. seeligeri* (Table 2.9) (Axelsson and Sorin 1998; Nadon *et al*. 2001; Nightingale *et al*. 2005; Kérouanton *et al.* 2010). All isolates are generally serotyped in accordance with the scheme for routine serodiagnosis of *L. monocytogenes* (Seeliger and Höhne 1979). Latex agglutination tests are commercially available for the identification of *L. monocytogenes* serotypes. Serotypic designation is on the basis of unique combinations of O and H antigens and their reaction with specific antisera.

Flagellar agglutination of serogroups 1/2 and 3 divides them into serovars while somatic agglutination characterizes serogroup 4 into serovars (Schönberg *et al*. 1996). There are 15 *Listeria* O antigen subtypes (I-XV) and the H antigens include four subtypes (A-D) (Jeyaletchumi *et al*. 2010) (Table 2.10). Serology is more often used for the identification and prevalence of *L. monocytogenes* serotypes for epidemiological studies and for the tracking of environmental contamination (Gasanov *et al*. 2005).

Of the 13 serotypes of *L. monocytogenes*, 1/2a, 1/2b and 4b are responsible for greater than 95% of listeriosis infections in humans, hence greater than 95% of isolates belong to only 3 serotypes. The genetic variation among these strains is mainly a result of genes encoding surface proteins, genes involved in sugar metabolism and virulence factors necessary for the infection of the host cell (Farber and Peterkin 1991; De Cesare *et al*. 2001; Cabrita *et al*. 2004; Dussurget *et al*. 2004; Bruhn *et al*. 2005; Neves *et al*. 2008). This sub-typing therefore indicates that some strains are more pathogenic to humans than others (McLauchlin 1987). A study undertaken by Nappi *et al*. (2005) showed that molecular characterization of *L. monocytogenes* by serotyping allowed for the identification of the strains 1/2a, 1/2b and 4b as the most important agents of invasive and non-invasive listeriosis in humans and ruminants.



However, most sporadic human cases and outbreaks have been caused by *L. monocytogenes* serotype 4b, suggesting specific virulence properties in this serotype (De Cesare *et al*. 2001; Cabrita *et al*. 2004; Doumith *et al*. 2004; Dussurget *et al*. 2004; de

Vasconcelos *et al*. 2008; Yde *et al*. 2010). Strains of the antigenic group 1/2 (1/2a, 1/2b, 1/2c) have been reported to predominate in food isolates but have been shown to be increasing in human isolates (Vázquez-Boland *et al*. 2001b; Cabrita *et al*. 2004). The genome of the *L. monocytogenes* serotype 4b isolate (CLIP 80459) has been partially sequenced and compared with the sequences of serotype 1/2a (EGDe) and *L. innocua*. Findings showed that there was a great genetic diversity within the *L. monocytogenes* species where 8% of the 4b serotype genes were absent from the serotype 1/2a genome and the latter, in turn, had 10.5% of its genes absent from the *L. innocua* genome. This emphasized that, although certain *L. monocytogenes* genes were absent from the *L. innocua* genome, it was obviously not required for the virulence of that particular *L. monocytogenes* strain. The work done also showed that genetic variation among *L. monocytogenes* serotypes is similar to that between *Listeria* species (Dussurget *et al*. 2004; Zhang *et al*. 2004a; Nightingale *et al*. 2005).



No links have been made between certain forms of listeriosis and specific serotypes, but work has shown that there is an association between perinatal listeriosis and serotypes 1/2a, 3b and 4b (Farber and Peterkin 1991). In Europe and North America, most listeriosis cases over the past 20 years involved serotype 4b and it was shown to be over-represented in perinatal listeriosis (Yde *et al*. 2010).

There are limitations when using serotying as a sub-typing tool for serotype identification. Serotyping measures the phenotypic characteristics of *L. monocytogenes*

and may give inconsistent results since it does not always accurately reflect the genotype of a microorganism (Liu 2006; van Belkum *et al*. 2007); however it is a very useful pre-requisite when used in conjunction with an alternative reproducible and reliable sub-typing method (Schönberg *et al*. 1996; van Belkum *et al*. 2007). The technique is also time consuming, difficult and requires high quality antisera (Dhanashree *et al*. 2003).

Furthermore, sequencing of a single gene may not be ideal for the accurate comparison and differentiation of *L. monocytogenes* isolates as the data may be uninformative if the gene is highly conserved or otherwise the data may be misleading if the gene is highly variable (Revazishvili *et al*. 2004).



**Table 2.9** Serotypes of *Listeria* spp.

| **SPECIES** | **SEROTYPE** |
|---|---|
| *L. monocytogenes* | 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7 |
| *L. innocua* | 3, 6a, 6b, 4ab |
| *L. ivanovii* | 5 |
| *L. seeligeri* | 1/2a, 1/2b, 1/2c, 4b, 4c, 4d, 6b |
| *L. welshimeri* | 1/2a, 4c, 6a, 6b |

**Table 2.10** Antigen sub-types of *Listeria* (adapted from Seeliger and Höhne 1979; Farber and Peterkin, 1991; Allerberger 2003)

| Serovar | O-antigens | | | | | | | | | | | | | | | | H-antigens | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1/2a | I | II | (III) | | | | | | | | | | | | | | A | B | | | |
| 1/2b | I | II | (III) | | | | | | | | | | | | | | A | B | C | | |
| 1/2c | I | II | (III) | | | | | | | | | | | | | | | B | | D | |
| 3a | | II | (III) | IV | | | | | | | | | | | | | A | B | | | |
| 3b | | II | (III) | IV | | | | | | | | | (XII) | (XIII) | | | A | B | C | | |
| 3c | | II | (III) | IV | | | | | | | | | (XII) | (XIII) | | | | B | | D | |
| 4a | | | (III) | | (V) | | | VII | | | IX | | | | | | A | B | C | | |
| 4ab | | | (III) | | V | VI | | VII | | | IX | X | | | | | A | B | C | | |
| 4b | | | (III) | | V | VI | | | | | | | | | | | A | B | C | | |
| 4c | | | (III) | | V | | | VII | | | | | | | | | A | B | C | | |
| 4d | | | (III) | | (V) | VI | | | VIII | | | | | | | | A | B | C | | |
| 4e | | | (III) | | V | VI | | | (VIII) | | (IX) | | | | | | A | B | C | | |
| 5[a] | | | (III) | | (V) | VI | | | (VIII) | | | X | | | | | A | B | C | | |
| 6a[a] | | | (III) | | V | (VI) | | (VII) | | | (IX) | | | | | XV | A | B | C | | |
| 6b[a] | | | (III) | | (V) | (VI) | | (VII) | | | IX | X | XI | | | | A | B | C | | |
| 7 | | | (III) | | | | | | | | | | XII | XIII | | | A | B | C | | |
| *L. grayi* | | | (III) | | | | | | | | | | XII | | XIV | | | | | | E |

Numbers in parentheses = variable.
[a]Serovars occur in non-*L. monocytogenes* species; all *L. ivanovii* are serovar 5.

### 2.5.2 Genetic sub-typing methods

#### 2.5.2.1 Pulsed Field Gel Electrophoresis (PFGE)

Since serotyping provides insufficient information in epidemiological investigations, highly discriminatory typing methods that correlate with serotyping are necessary. In 1984, Schwartz and Cantor described PFGE as a new way to separate very large DNA molecules which would circumvent the limitations of fractionating these large DNA molecules by standard gel electrophoresis (Joppa *et al*. 1992). This sub-typing technique is considered the 'gold standard' for epidemiological investigations (Wiedmann 2002; Hunter *et al*. 2005; Neves *et al*. 2008). The chromosomal DNA of an isolate is digested by a restriction enzyme and the DNA fragments are separated according to size by agarose gel electrophoresis (Joppa *et al*. 1992). PFGE has shown to have excellent discriminatory power and reproducibility and very useful for molecular sub-typing of *L. monocytogenes* (Miettinen *et al*. 1999; Yde and Genicot 2004; Ducey *et al*. 2007).



Conventional DNA gel electrophoresis allows for the separation of DNA molecules up to 50 kb, whereas PFGE allows for the separation of very large molecules up to 12 Mb (Joppa *et al*. 1992; Gasanov *et al*. 2005; Herschleb *et al*. 2007). During standard electrophoresis, a constant electrical field enables fractionated DNA molecules to be separated in a size dependant manner through a solid gel matrix, namely agarose or polyacrylamide. However, limitations do occur during conventional electrophoresis;

DNA larger than 30-50 kb migrates with the same mobility in spite of size, which in turn is observed as one large band on the gel. This occurs since very large DNA molecules are present as soft random coils which only after sufficient unraveling (where they elongate and align themselves with the electric field) will electrophorese through a tight gel matrix. Large and small molecules travel through the gel matrix except there is no indication of size-dependant mobility. However, where the DNA is forced to change direction or reorient, fragments that differ in size will separate from each other on the basis that when the electric field is altered periodically, smaller sized DNA will reorient and migrate more quickly than the larger DNA through the pores in an agarose gel. The periodic change in the direction of the electric field forces the DNA molecules in the gel to relax when the first electric field is removed and then elongate to align to the new field. The altering electrical pulses facilitate the separation of DNA molecules in a size dependant manner (Joppa *et al*. 1992; Herschleb *et al*. 2007).



PFGE is a valuable tool for genomic DNA analyses where applications include the detection of large-scale genetic polymorphisms and a strain-tying method for pathogenic micro-organisms. Relevant information on genome size and constructing physical and genetic maps of chromosomes of bacteria have been obtained by PFGE (Basim and Basim 2001).

A limitation of other sub-typing techniques is that they are unable to sub-type *L. monocytogenes* serotype 4b whereas PFGE is very useful for the sub-typing of this serotype (Jeyaletchumi *et al*. 2010).

Various types of PFGE exists, two of which include Contour-Clamped Homogeneous Electric Fields (CHEF) and Transverse-Alternating Field Gel Electrophoresis (TAFE). CHEF is the most widely used apparatus (Basim and Basim 2001). It has two unique properties which includes highly uniform electric fields which it provides within the gel as a result of 24 electrodes equally spaced around a hexagonal contour which eliminates lane distortion and secondly the CHEF system generates a 120°C re-orientation (field) angle which is optimal for separating DNA molecules ranging from 100 kb to 6 Mb in size (Bio-Rad Laboratories 1992; Basim and Basim 2001).



TAFE is another form of PFGE whereby the gel is positioned vertically and a four electrode array is placed in the front and back of the gel. The molecules move in a zigzag motion down the gel where all lanes equally experience continuous variations in field strength and reorientation angle. Molecules however do not move with a constant velocity over the length of the gel as there is a difference between the angles of the electric field at the top of the gel (115°C) to the bottom of the gel (165°C) (Basim and Basim 2001).

### 2.5.2.2 PCR-based sub-typing techniques

#### 2.5.2.2.1 PCR-RFLPs

PCR-RFLPs have been used for sub-typing different micro-organisms and in some instances the DNA patterns may be used for organism identification (Koneman *et al*. 2006). PCR-RFLP analysis involves PCR amplification of housekeeping or virulence associated genes (e.g. hemolysin - *hly*) which are then subsequently digested with specific restriction enzymes (Liu 2006). The target gene would have polymorphisms which would facilitate discrimination within *L. monocytogenes*. The polymorphism would be detected once agarose gel electrophoresis is employed and a specific banding pattern would result on account of the polymorphism being present (Jeyaletchumi *et al*. 2010). Banding patterns of fragments of varying sizes are produced based on restriction enzyme digestion of the target gene which usually codes as a virulence factor (Gasanov *et al*. 2005). This technique provides a sensitive and reproducible approach for sub-typing *L. monocytogenes* into lineage groups and is more discriminatory when used in conjunction with other sub-typing techniques. PCR-RFLPs have been applied as a molecular sub-typing tool in other studies as well (Moorhead *et al*. 2003; Ueda *et al*. 2005).



A useful tool for differentiating between strains is based on single nucleotide polymorphisms (SNP). The simplest type of polymorphism, namely SNP, results from a

mutation in a single base which is then substituted by another nucleotide. SNPs refer to a change in a single nucleotide that can occur in a DNA sequence (Schork *et al*. 2000). For example, when comparing a conserved region in a gene in a number of serotypes and there is a variation in a single nucleotide where nucleotide T replaces one of the other 3 nucleotides, namely; C, A, or G, then T is considered the SNP (Figure 2.5). The digestion of a piece of DNA, containing the SNP, with the relevant restriction enzyme could distinguish alleles or variants as a result of the fragment sizes generated via gel electrophoresis. This type of polymorphism is termed RFLPs (Schork *et al*. 2000). One approach to write SNPs is the nucleotide followed by the SNP location and base substitution e.g G719T refers to nucleotide G being substituted by T at the nucleotide position 719 in the gene.



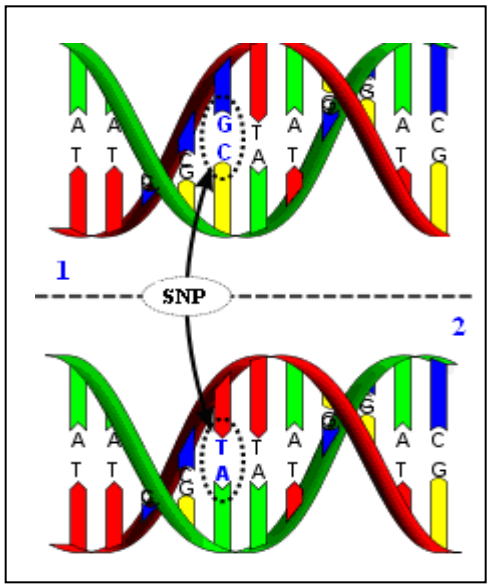



**Figure 2.5** A SNP occurring in a DNA molecule. A (G/T) polymorphism between molecule one and two (adapted from Hall 2007).

## 2.6 PATHOGENICITY

*Listeria monocytogenes* is an intracellular pathogen that can invade and replicate in epithelial cells and macrophages. It is capable of crossing three barriers namely the intestinal, blood-brain and placental barriers (Dussurget *et al*. 2004; Lecuit 2005; Seveau *et al*. 2007). The fact that *L. monocytogenes* can cause severe illness is due to its ability to induce its own uptake by the host cell and then replicate and spread to other cells. This process is facilitated by the bacterium producing a series of virulence factors for each step of the invasion process (McLauchlin 1987; Greiffenberg *et al*. 2000; Doyle 2001; Vázquez-Boland *et al*. 2001a). A transcriptional activator PrfA activates all the virulence genes on the cluster and regulates their expression (Chatterjee *et al*. 2006).



### 2.6.1 Virulence factors

#### 2.6.1.1 *Listeriolysin O and phospholipases*

The ability of *Listeria* to invade and replicate in host cells depends on its virulence genes (Ragon *et al*. 2008). The virulence determinants of *Listeria* spp. are clustered along the chromosome in genomic islands. The locus for *L. monocytogenes* consists of 3 transcriptional units. First is the central position - the *hly* monocistron; downstream from *hly* is an operon comprising 3 genes namely, *mpl*, *actA*, *plcB* and upstream from *hly* is the *plcA*-*prfA* operon (Vázquez-Boland *et al*. 2001a).

The hemolysin gene, *hly*, was the first virulence factor to be recognized and sequenced in *Listeria* spp. and its specific role in the pathogenesis of *Listeria* infection was demonstrated (Farber and Peterkin 1991; Vázquez Boland *et al*. 2001b; Seveau *et al*. 2007).

Early evidence that the *Listeria* hemolysin (*hly*) is similar in function and antigenicity to streptolysin O (SLO) from *Streptococcus pyogenes* was provided. Eventually it was confirmed that the hemolysin of *L. monocytogenes* is a SLO-related cytolysin and a cholesterol-dependent pore-forming toxin (Vázquez-Boland *et al*. 2001b). This toxin was designated listeriolysin O (LLO).



As *L. monocytogenes* invades the host cell, they reside in the vacuole that is surrounded by a membrane. *L. monocytogenes* will almost immediately be killed by phagocytic cells unless they are able to produce LLO and thereby lyse the vacuole and escape into the cytoplasm. LLO is a 60-kDa protein that allows *L. monocytogenes* to escape from the vacuole (Dussurget *et al*. 2004; Jiang *et al*. 2006). Mutants that lack LLO are unable to reach the cytoplasm. The absence of *hly* therefore equals avirulance (Doyle 2001; Vázquez-Boland *et al*. 2001a; Pushkareva and Ermolaeva 2010) thus it can be said that LLO is secreted by all virulent strains of *Listeria*. Once in the cytoplasm, the organism will acquire nutrients from the cytosol and multiply (Figure 2.6). *L. monocytogenes* secretes two Phospholipase Cs (PLCs) that are implicated in the lysis of intracellular vacuoles. They act with LLO in facilitating the lyses of primary and secondary vacuoles

(Camilli *et al*. 1993). The first PLC is phospatidylinositol (PI-PLC) specific and the second PLC is phosphatidylcholine (PC-PLC) specific. Studies have showed that PI-PLC assists in the escape of the organism from the primary vacuole whereas PC-PLC is active during cell-to-cell spread of the bacteria (Doyle 2001).

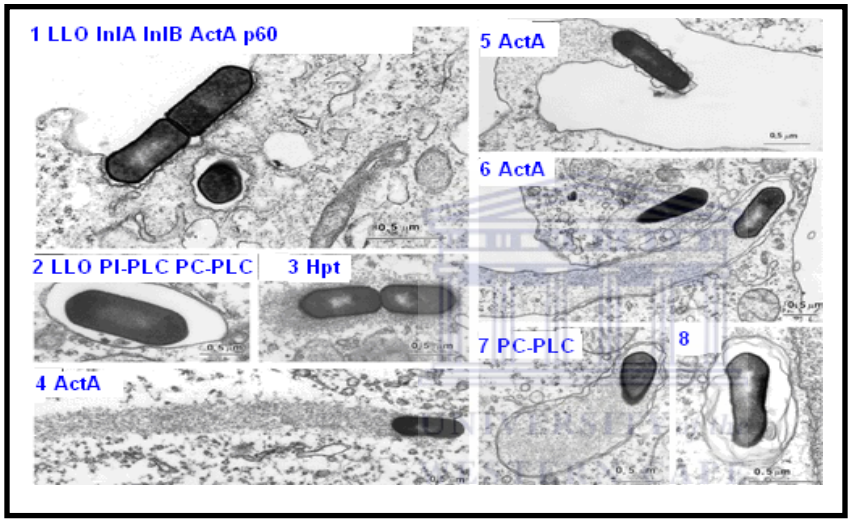



**Figure 2.6** An electron micrograph showing how *L. monocytogenes* enters and lyses the host cell with the aid of virulence factors at each step. **1** entry, **2** lysis of the vacuole, **3** intracellular replication, **4** intracellular movements, **5;6** cell-to-cell spread, **7** formation, **8** lysis of the two-membrane vacuole (adapted from Dussurget *et al*. 2004).

### 2.6.1.2 *Actin Polymerizing Protein A* (ActA)

Downstream from *hly* is a 5.7kb operon comprising *actA* (1 of 3 genes). The gene *actA* encodes the surface protein ActA (Vázquez-Boland *et al*. 2001a). Once the vacuole has been lysed and *L. monocytogenes* reaches the cytoplasm, they need to reach other cells in

order for them to multiply. They do this by means of ActA, a 639 amino acid protein that induces polymerization of globular actin molecules to actin filaments, which allows the bacterium to move from cell to cell along these filaments to the cell membrane (Chatterjee *et al*. 2006; Schuppler and Loessner 2010). Hence, ActA allows for the mobility of *L. monocytogenes* and attachment and entry into target cells (Kocks *et al*. 1992; Doyle 2001; Jiang *et al*. 2006; Seveau *et al*. 2007). ActA is therefore also required for *L. monocytogenes* pathogenicity (Vázquez-Boland *et al*. 2001a). In a study undertaken by Doyle (2001) to compare the virulence of *L. monocytogenes* serotypes in mice, lower virulence was observed by serotypes 4a, 4c, 4d and 4e. This was attributed to the production of lower levels of the ActA protein with actin tail formation. In other similar experiments, low levels of LLO and internalin (Inl) was observed (Doyle 2001).



#### 2.6.1.3 *Protein p60* (p60)

The *iap* gene encodes the extracellular protein p60, which is common to all *Listeria* spp. (Kohler *et al*. 1990). It is regarded as an essential murein hydrolase enzyme that facilitates septum separation during the final stage of cell division (Jiang *et al*. 2006). It has been shown that p60 plays a role in the adherence of the bacterium to the host cell (Bubert *et al*. 1999) and is involved in *L. monocytogenes* virulence and pathogenicty (Jeyaletchumi *et al*. 2010). The *iap* gene was demonstrated to be a reliable PCR target for differentiation of *Listeria* spp. It has conserved regions at the 5' and 3' ends and a species-specific internal region (Cocolin *et al*. 2002).

#### 2.6.1.4 *Positive Regulatory factor A* (PrfA) *Regulon*

Important *Listeria* virulence genes including, *hly*, *actA*, *prfA*,, hexose phosphate transporter (*hpt*), metalloprotease (*mpl), plcA* and *plcB* are regulated by a transcriptional activator PrfA, a protein comprising 233 amino acids. They are known as the PrfA-dependent virulence gene cluster. PrfA is the only regulator identified to date in *Listeria* spp. which is directly involved in the control of virulence gene expression within infected host cells (Table 2.11) (Vázquez-Boland *et al*. 2001a; Dussurget *et al*. 2004). The internalin (*inlA*, *inlB*) genes are positioned elsewhere in the genome and have a transcription binding site similar to that recognized by PrfA; as a result, they may be to some extent regulated by PrfA (Jeyaletchumi *et al*. 2010). The virulence gene cluster, which is present in *L. monocytogenes*, has been shown to be completely absent from 3 other non-pathogenic serotypes of *Listeria* namely *L. innocua*, *L. welshimeri* and *L. grayi* (Vázquez-Boland *et al*. 2001a).

**Table 2.11** Virulence genes regulated by the PrfA Regulon

| Virulence Genes | Function | Reference |
|---|---|---|
| ***prfA*** | This gene encodes PrfA, a protein which is necessary to activate all the genes of the cluster (located in a 9.6 kb region) and vital to the virulence potential of *L. monocytogenes.* | Ward *et al*. 2004; Domínguez-Bernal *et al*. 2006; Jeyaletchumi *et al*. 2010 |
| ***inlA* and *inlB*** | InlA is a bacterial surface protein encoded by the gene *inlA* that plays a role in the crossing of the intestinal barrier. InlB is a protein encoded by the gene *inlB* and acts both as a growth factor and as an invasin. It plays a role in the invasion of hepatocytes in the liver. InlA and InlB are a family of leucine-rich repeat proteins. They were the first factors associated with the invasion of the target cell.<br><br>Almost all isolates causing human listeriosis have a full-length functional InlA whereas those with a truncated form are adapted to food and the environment and are associated with a reduced virulence potential. | Braun *et al*. 1998; Greiffenberg *et al*. 1998; Doyle 2001; Hain *et al*. 2006; Seveau *et al*. 2007; Schuppler, M. and Loessner 2010;<br><br>Ragon *et al*. 2008 |
| ***Hpt*** | *Hpt* encodes a hexose phosphate transporter (Hpt). It functions as a sugar uptake system that allows bacterial intracellular replication. *L. monocytogenes* uses phosphate sugar in the cytoplasm to obtain nutrients from the host cell. | Dussurget *et al*. 2004; Domínguez-Bernal *et al*. 2006 |
| ***Mpl*** | The *mpl* gene encodes an enzyme metalloprotease (Mpl) that processes the immature form of PC-PLC into a mature form. It works with *hly*, *plcB* and *plcA* to disrupt the primary vacuoles after host cell invasion. Mutations in *mpl* have shown to reduce virulence in a mouse model. | Dreverts 1998; Vázquez- Boland *et al*. 2001a; Todar 2008; Schuppler, M. and Loessner 2010 |
| ***plcA*** | *plcA* encodes the protein PlcA (a PI specific PLC). This protein works with *hly* and *plcB* to disrupt 1º vacuoles. | Mengaud *et al*. 1991; Jeyaletchumi *et al*. 2010 |
| ***plcB*** | *plcB* encodes the protein PlcB (a PC specific PLC). Its primary function is to disrupt the double membrane 2° phagosomes formed after cell to cell spread. The 3 protein products from the *mpl-actA-plcB* operon all assist in cell to cell spread of *L. monocytogenes* and its escape from the host's immune response in the extracellular compartment. | Dreverts 1998; Vázquez- Boland *et al*. 2001a |

### 2.6.2 Entry into host cells

The susceptibility of the host plays a major role in the manifestation of disease upon exposure to *Listeria monocytogenes*. *L. monocytogenes* is seen as an opportunistic disease since most listeriosis patients have a physiological or pathological defect that affects T-cell-mediated immunity facilitating pathogen invasion (Vázquez-Boland *et al*. 2001b). As noted previously, *L. monocytogenes* serotypes 1/2a, 1/2b and 4b are responsible for greater than 95% human listeriosis cases indicating an increase in virulence potential in these strains. Phylogenetic analyses has shown that *L. monocytogenes* exists in three evolutionary lineage groups namely, lineage I, II and III. The pathogenic potential of lineage group I is considered high, lineage II medium and lineage III low (Bhunia 2008). Lineage I consists of serotypes belonging to flagellar antigen type b and d namely; 1/2b, 3b, 4b, 4d and 4e and lineage II consists of serotypes belonging to flagellar antigen type a or c namely; 1/2a, 1/2c, 3a or 3c except serotypes 4a and 4c (Nadon *et al*. 2001; Tran and Kathariou 2002; Schmid *et al*. 2003; Ragon *et al*. 2008). Lineage I isolates appear to be over-represented among human listeriosis cases while lineage II isolates show an over-representation among food and environmental samples and fewer in human disease (Liu *et al*. 2006; Fugett *et al*. 2007; den Bakker *et al*. 2008). Serotypes 4a and 4c, which are rather uncommon, have been characterized as lineage III isolates due to its genetic distance from other serotypes and that it is rarely implicated in listeriosis but mainly associated with animal infections (Zhang *et al*. 2003; Roberts *et al*. 2006; den Bakker *et al*. 2008). Ribotyping and virulence gene

polymorphism data were used to describe the existence of this third lineage (Ward *et al*. 2004). A very negligible number of *L. monocytogenes* serotype 4a and 4c strains originate from human listeriosis cases which is suggestive of an absence of virulence associated transcriptional regulator genes that encode invasion associated proteins required to complete the infection cycle in the host (Liu 2006). Lineage III strains were shown to have unique 16S rRNA sequences which were lacking in strains of lineage I and II (Jeffers *et al*. 2001). Lineage III (4a and 4c) can be further divided into two groups namely IIIA and IIIB. Isolates of lineage IIIB are generally rhamnose-negative although *L. monocytogenes* is rhamnose-positive. Even though these strains closely resemble *L. innocua*, they are not classified as such due to the presence of *L. monocytogenes* virulence genes *actA*, *hlyA* and *inlA* (Wiedmann 2002).



In a study undertaken by den Bakker *et al*. (2008) to determine the rate of recombination between lineage groups of *L. monocytogenes*, lineage I isolates (responsible for most sporadic and epidemic human listeriosis cases) appeared to have the lowest recombination rate. Lineage II isolates had a high recombination rate and the authors suggest that this may be necessary for its survival and competition in a broad range of different environments since this lineage group is over-represented in food and environmental samples.

Subtype discrimination by ribotyping showed that the subtypes common in foods in France and the US displayed a reduction in their competency to invade human intestinal

epithelial Caco-2 cells. This was a result of mutations leading to premature stop codons in the *inlA* gene. These mutations in *inlA* would explain the attenuated human virulence by these strains. *InlA* with premature stop codons are represented among much food isolates which supports why many food isolates show reduced virulence (Oliver *et al*. 2007). Attenuated human virulence, but to a lesser degree, may occur as a result of mutations in other virulent genes such as *hly* and *prfA* (Oliver *et al*. 2007). Most food isolates are characterize as lineage II strains and strains of this lineage appear less likely to cause human disease as compared to lineage I strains (Oliver *et al*. 2007).

In a study undertaken in Spain to determine the prevalence and strain diversity of *L. monocytogenes* in healthy ruminants and swine herds, serotyping of 114 isolates characterized 84.2% as serotype 4b and 13.2% as serotype 1/2a (Esteban *et al*. 2009).



Serotype 4b is mainly of concern since it is largely implicated in sporadic and epidemic human listeriosis outbreaks. The clinical manifestation of illness with this serotype is usually more severe and it is associated with a higher mortality rate (Ward *et al*. 2010). It also includes three epidemic clones (ECs) which have been responsible for numerous outbreaks worldwide (Ducey *et al*. 2007; Chen and Knabel 2008; Ward *et al*. 2010). A source strain that is the cause of an outbreak may result in the start of an epidemic. The source strain may persist and cause illness over a long period of time which results in an epidemic. An EC can be defined as a group of isolates part of the 'same chain of transmission' and are descendants of the source strain (referred to as the clone) (Chen *et*

*al*. 2005). ECI, II and IV refer to serotype 4b clusters that were linked to outbreaks associated with the ingestion of contaminated foods of which ECI was linked to coleslaw, soft cheese and pork; ECII to hotdogs and turkey deli meats and ECIV to pâté and vegetables (Chen and Knabel 2008). In Europe and North America, serotype 4b was reported to be responsible for all epidemic outbreaks for the two decades prior to this reporting (Schmid *et al*. 2003).

#### 2.6.2.1 *Invasion of the intestine*



Once *Listeria monocytogenes* is ingested with food, it first has to cross the intestinal wall before severe symptoms of listeriosis is manifested (Doyle 2001). *L. monocytogenes* enters with the aid of p60 and internalins, which facilitates its attachment to intestinal walls. A study undertaken with rodents showed that a point of entry for *L. monocytogenes* was Peyer's patches lining the intestine, where they were found to multiply rapidly. They were then carried in macrophages or dendritic cells through the lymphatic system to mesenteric lymph nodes and then via the blood stream to the liver, spleen, placenta and central nervous system (Chen *et al*. 2000; Doyle 2001) (Figure 2.7a and Figure 2.7b).

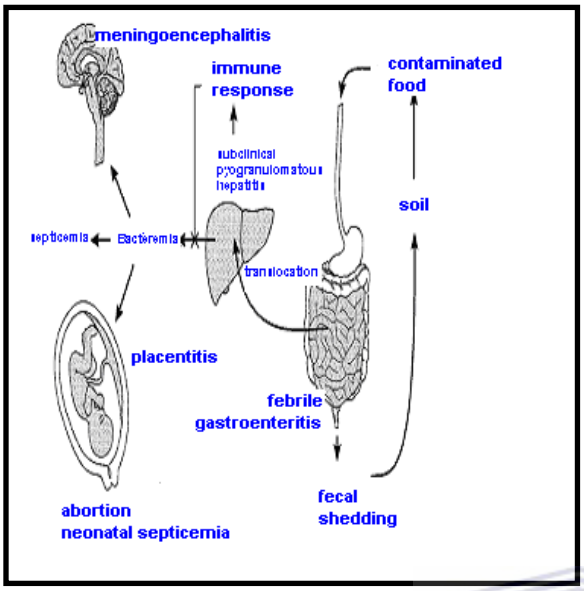



**Figure 2.7a** *L. monocytogenes* infection cycle. The pathogen is capable of crossing three barriers, namely the intestinal, blood-brain and placental barrier (adapted from Vázquez-Boland *et al*. 2001b).

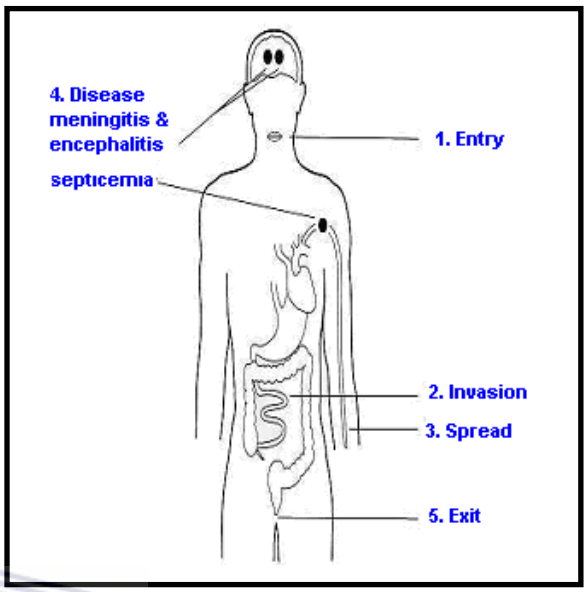



**Figure 2.7b** The pathogenicity of *L. monocytogenes*. Infection results in two main clinical manifestations, namely sepsis and meningitis (adapted from Hof, accessed April 26, 2011).

### 2.6.2.2 *Invasion of the liver*

As noted above, the *Listeria monocytogenes* that cross the intestinal barrier are carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver. If an adequate immune response is not elicited in the liver, then *L. monocytogenes* will proliferate which will facilitate its invasion/spread to the bloodstream (Vázquez-Boland *et al*. 2001b) and cause septicemia.

#### 2.6.2.3 *Invasion of the placenta*

Pregnant women represent a high risk-group for *Listeria monocytogenes* infection (Abram *et al*. 2003). The risk of acquiring infection is 20 times greater compared to healthy individuals (Curtis 2007). *L. monocytogenes* targets and crosses the placental barrier in pregnant women. The crossing of the placental barrier leads to serious fetal infections, fetal death, miscarriages, premature births and neonatal infections (Longhi *et al*. 2003; Todar 2008; Schett *et al*. 2005). The interaction between a protein from *L. monocytogenes*, internalin, and its host's cell receptor, E-cadherin (a transmembrane protein expressed by epithelial cells), has been shown to facilitate entry to the human placental barrier (Lecuit *et al*. 2004; Dominguez-Bernal *et al*. 2006). Macrophages in the body that contain *Listeria* and enter the placenta infect endothelial cells and then the fetus resulting in premature labour or death of the fetus, otherwise sepsis or meningitis will result if the infant is infected while passing through the birth canal (Doyle 2001). The mother usually survives (Curtis 2007). Serovar analysis from patients identified serotype 4b as being the most prevalent in pregnancy-associated cases (Doyle 2001). Most published outbreaks in Europe and North America show the over-representation of serotype 4b linked to perinatal listeriosis which may indicate that this serotype has an increased virulence for pregnancy associated cases whereby it can cross the placental barrier (Yde *et al*. 2010)

Health authorities in the UK advise pregnant women to abstain from eating foods that have an increased risk associated with illness. These foods include soft mould-ripened or blue veined cheese, pâté and unpasteurized dairy products. They may also choose to avoid cold RTE meats and smoked salmon, to heat RTE meats adequately and wash RTE salads thoroughly (Curtis 2007).

#### 2.6.2.4 *Invasion of the brain*

*Listeria monocytogenes* is able to invade endothelial cells and is known to cause meningitis and encephalitis in infected individuals resulting in a high mortality rate (Schuchat *et al*. 1991; Greiffenberg *et al*. 1998). A study undertaken by Greiffenberg *et al*. (2000) showed that invasion of the human brain microvascular endothelial cells (HBMEC) with *L. monocytogenes* was dependant on the *inlB* gene. An electron micrograph showed that *L. monocytogenes* invades the HBMEC and that intracellular multiplication, movement and production of bacterium containing protrusions accompany it. The fact that *L. monocytogenes* can invade HBMEC, illustrates that it is able to cross the blood-brain barrier (Figure 2.8).

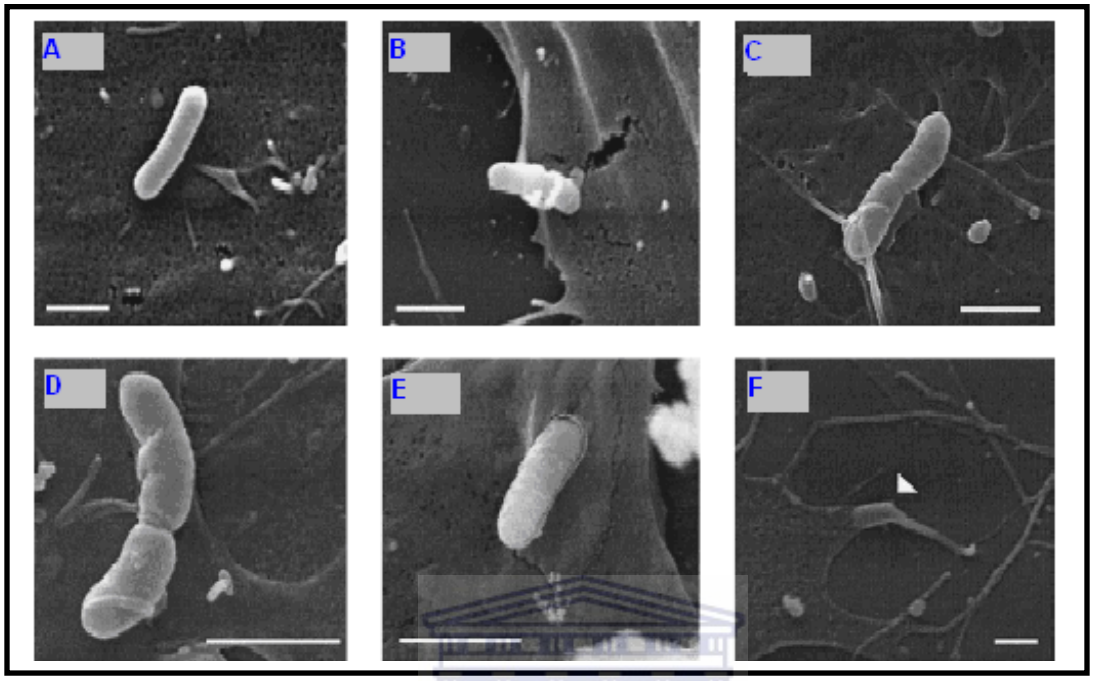



**Figure 2.8** An electron micrograph showing a 35 minute postinfection of HBMEC with *L. monocytogenes*. **A** *L. monocytogenes* on the cell surface or; **B, C, D** in contact with microvilli; **E** rarely was *L. monocytogenes* seen in the process of invasion or; **F** already taken up by the HBMEC (arrowhead) Bars 1µm (adapted from Greiffenberg *et al*. 2000).

## 2.7 DETECTION METHODS FOR *LISTERIA*

### 2.7.1 Conventional (traditional) methods

Conventional methods for microbial detection require the target organism to grow and form colonies on a specific growth medium. These methods, although fairly cheap, are labour intensive with regard to media preparation and recording the results as well as

time consuming (a time period of 5-7 days to complete) (Norton and Batt 1999; Choi and Hong 2003; Gouws and Liedemann 2005). Conventional methods are also unreliable especially for thermally injured or stressed organisms (Norton and Batt 1999). Conventional methods for detecting micro-organisms require several stages namely dilution, pre-enrichment, selective enrichment broth, selective plating and biological and/or serological tests (Lantz 1998; Purwati *et al*. 2001; Neamatallah *et al*. 2003). However, the need for traditional microbiological methods should not be underestimated or overlooked where viable isolates are required for sub-typing methods used for outbreak investigations.




**2.7.1.1** ***Diluting a sample***

Homogenizing a food sample such as diluting the material generates a large volume of material. The distance between the inhibitors and target molecule is increased, thereby reducing the chance of interference of the inhibitor with the target (Tsai and Olen 1992). Diluting a sample has been shown to be effective when aiming to reduce the effect of inhibitors on the PCR reaction (Fredericks and Relman 1998). In a study undertaken by Fredericks and Relman (1998), the inhibitory effect of sodium polyanetholesulfonate (SPS), an additive to blood culture medium, could only be overcome by diluting the samples. Where low numbers of the target cell is present, diluting a sample will only reduce the bacterial numbers even more, therefore an enrichment step should be

performed before a dilution is carried out to ensure that detection of the target cell is allowed.

#### 2.7.1.2 *Pre-enrichment*

The recovery of bacterial pathogens from foods including raw milk, dairy products and meat can be complicated due to the presence of high numbers of indigenous microflora and other pathogens and because the pathogens of interest may be sub-lethally injured at the time of testing. Enrichment in nonselective (pre-enrichment) and selective media is usually required for the detection and identification of food-borne pathogens. The recovery of *Listeria* spp. from food requires the use of selective enrichment media such as *Listeria* enrichment broth (LEB), containing antibiotics that are inhibitory to competitive microorganisms. Much effort has been employed to find an enrichment media and protocol for *L. monocytogenes* and other *Listeria* spp. isolation. An ideal enrichment medium would promote the recovery and proliferation of *L. monocytogenes* and *Listeria* spp. over other microflora (Cocolin *et al*. 2002). Pre-enrichment or primary enrichment broths generally have smaller amounts of selective agents, which promote the recovery of injured/stressed cells (Beumer and Hazeleger 2003). With conventional detection methods, false positives may arise as a result of other *Listeria* spp. being present with *L. monocytogenes*. Overgrowth of *Listeria* spp. may also result during the enrichment procedure resulting in *L. innocua* out-competing *L. monocytogenes* (Norton *et al*. 2001; Willis *et al*. 2006) and mimicking the appearance of *L. monocytogenes* on a

culture medium. For this reason further testing to differentiate between species needs to be employed. The function of the enrichment steps prior to the PCR reaction increases the number of target cells (after 4 h the bacteria should double in numbers) and secondly it facilitates the PCR reaction by reducing false negative results (Ericsson and Stålhandske 1997; Duffy *et al*. 2001).

*Listeria monocytogenes* may be sub-lethally stressed or injured when present in a food product due to extreme temperature or pH conditions and in certain cases, selective agents present in selective enrichment media may interfere with the repair of these cells, since selective media contain agents which select for healthy target organisms. These cells, which are then undetected, can recover and grow during the storage of food (Pearson and Marth 1990; Wu and Fung 2001; Yuste *et al*. 2003; Rijpens and Herman 2004; Gasanov *et al*. 2005). Since food samples and enrichment media can be inhibitory to the PCR reaction, it may be necessary to subculture in a non-selective medium prior to the PCR reaction (Gouws and Liedemann 2005). In some cases the enumeration of *L. monocytogenes* from food products was greater when a short, non-selective enrichment step was employed rather than a complete, long selective enrichment step (Rijpens and Herman 2004). In a study undertaken by Rijpens and Herman (2004), results showed that when a non-selective enrichment step was employed (using buffered peptone water (BPW)), the recovery rate of *L. monocytogenes* increased within some cheeses. This non-selective enrichment step was either employed for 5 or 24 h followed by a shorter selective enrichment step, compared to one long selective enrichment step.

#### 2.7.1.3 *Selective enrichment broth*

By using Fraser broth, optimum growth conditions are created for *Listeria* due to the high nutrient content and the large buffering capacity, which enhances cell growth and repair. The growth of accompanying bacteria is largely inhibited by selective agents - lithium chloride, nalidixic acid and acriflavine hydrochloride. The glucose esculin is cleaved by β-D-glucosidase into esculetin and glucose. The esculetin then forms an olive-green to black complex with the iron (III) ions resulting in a blackening of the broth indicating the presence of *Listeria* spp. (Gasanov *et al*. 2005; Oxoid manual http://www.oxoid.com/uk/index.asp)



#### 2.7.1.4 *Isolation through selective plating and chromogenic media*

*Listeria* cells tend to grow slowly and are outgrown by other competitors. For this reason selective agents such as acriflavin and nalidixic acid have been added to selective agar or enrichment broths to facilitate the proliferation of *Listeria* while suppressing competing microflora in food and environmental samples (Gasanov *et al*. 2005). Selective plating represses the growth of some organisms so others will grow. This is achieved by adding inhibitors or imposing certain conditions. Oxford and PALCAM agar are selective for *Listeria* based on esculin hydrolysis; however it does not differentiate *L. monocytogenes* from the rest of the species (Neamatallah *et al*. 2003; Marrakchi *et al*. 2005). In a study undertaken by Marrakchi *et al*. (2005), Oxford agar was shown to be more effective than

PALCAM agar for the isolation of *L. monocytogenes* from marine samples; however a limitation that it does not distinguish *L. monocytogenes* from the other spp. of its genus exists, especially important for the recovery of *L. monocytogenes* from food products. It is therefore evident that these conventional detection methods are time consuming and laborious since subsequent tests need to be performed in order to differentiate between species.

Oxford agar formulation is based on Columbia agar to which lithium chloride, acriflavin, colistin sulfate, sefotetan, cycloheximide and fosfomycin have been added. These ingredients suppress the growth of Gram - negative bacteria and the greater part of Gram - positive bacteria. β-D-glucosidase hydrolyses esculin into esculetin and forms a black complex with iron (III) ions. Therefore, *L. monocytogenes* produces grey-green coloured colonies with a black halo (Merck manual 1996; Willis *et al*. 2006). Some other organisms that are able to utilize esculin example, *Bacillus* spp. and *Enterococcus* may mimic the appearance of *Listeria* spp. therefore further tests may need to be performed in order to confirm presumptive results (Gasanov *et al*. 2005).



PALCAM (Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol) agar provides selective isolation of *Listeria* while at the same time inhibiting the Gram-negative and most of the accompanying Gram-positive bacteria. Mannitol and a pH indicator, phenol red, have been added to differentiate mannitol-fermenting strains from *Listeria* based on mannitol fermentation. Mannitol fermentation is demonstrated by a

colour change in the colony and/or the surrounding medium from red to grey to yellow due to the production of acidic end-products (Difco manual 1998). The media comprises polymixin, acriflavin, ceftacidim and lithium chloride. *L. monocytogenes* breaks down the esculin in the medium to esculetin and glucose. Esculetin forms an olive-green to black complex with iron (III) ions, which stains the colonies of *L. monocytogenes* (Merck manual 1996). Colonies of *Listeria* appear grey-green with a black precipitate after inoculation and incubation at 35°C for 24-48 h (Allerberger 2003).

The rapid identification of *Listeria* in food products is very important in order to protect consumers from infection. Various chromogenic media have been developed to allow for the distinction between the pathogenic *L. monocytogenes* and other *Listeria* spp. The development of chromogenic media such as RAPID'L.mono agar (Biorad) and chromogenic Precis *Listeria* (Oxoid) has allowed for the rapid detection of *L. monocytogenes.* This technique is facilitated by the identification of bacterial enzymes by chromogenic substrates in the media which causes the bacterial colonies to have a characteristic colour (Gasanov *et al*. 2005). The distinction is on the basis of enzymes produced as well as acid production due to the fermentation of sugars (Jeyaletchumi *et al*. 2010). On chromogenic RAPID'L.mono, blue colonies are indicative of a positive result for *L. monocytogenes* whereas on *Listeria* Precis media, green-blue colonies surrounded by a halo are indicative of a positive result for *L. monocytogenes*. Chromogenic media differentiates between *Listeria* spp. based on the PI-PLC activity (Gasanov *et al*. 2005; Czuprynski *et al*. 2010) that is present in *L. monocytogenes*, *L. seeligeri* and *L. ivanovii*

but not the other *Listeria* spp (Jemmi and Stephan 2006). PI-PLC positive bacteria produce blue colonies on RAPID'L.mono (Gasanov *et al*. 2005). Blue colonies with a yellow halo are indicative of *L. ivanovii*. Colonies that are white to pale yellow indicate *Listeria* spp. other than *L. monocytogenes* (Figure 2.9).

The chromogenic Precis *Listeria* (Oxoid) media is designed to detect all *Listeria* species as well as to distinguish *L. monocytogenes* and *L. ivanovii* from the non-pathogenic species. An enzyme, β-glucosidase cleaves the chromogen to produce green-blue colonies. Furthermore, soya lecithin is hydrolyzed by enzymes of *L. monocytogenes* and *L. ivanovii* which results in the production of an opaque halo around the colony while other *Listeria* species produce green-blue colonies with no halo (Willis *et al*. 2006). These chromogenic media allow presumptive identification within 24 h.



Compared to selective agars, non-selective agars allow for the growth of non-injured and sub-lethally stressed organisms but allow no differentiation within the population (Wu and Fung 2001). On general purpose media (agar), *L. monocytogenes* forms small, translucent colonies in 24-48 h when incubated at 37°C aerobically or anaerobically.

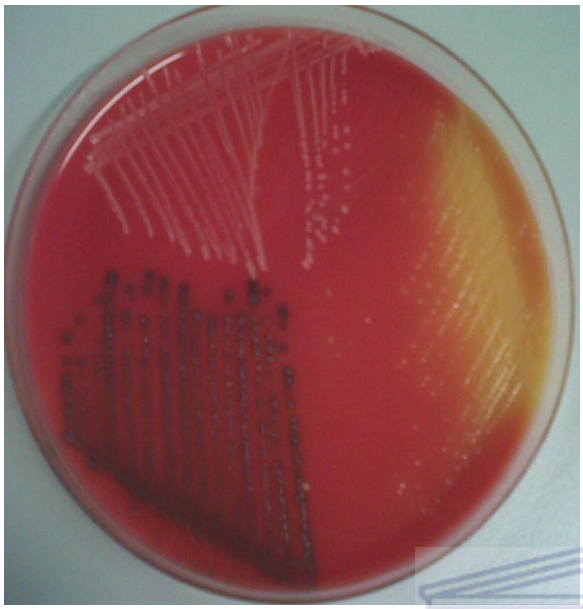


**Figure 2.9** *Listeria* spp. on RAPID'L.mono agar.



### 2.7.1.5 Haemolysis

Partial differentiation between *Listeria* spp. occur based on haemolytic activity whereby *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* produces varying zones of haemolysis. A zone which resembles a halo, surrounds the colony (Jeyaletchumi *et al*. 2010). *L. monocytogenes* produces a small zone of haemolysis, *L. innocua* produces no zone of haemolysis and *L. ivanovii* produces a clear zone of haemolysis. The zones of haemolysis can be examined by allowing bright light to shine through the back of the plate.

#### 2.7.1.6 *Gram Stain and API- Listeria*

For further confirmation and identification, microscopy can be employed to differentiate between a Gram-positive and Gram-negative organism. *Listeria* are Gram-positive, slim, short and rod-shaped. The API-Listeria (BioMérieux), which includes ten tests, relies on biochemical analysis that is based on the fermentation of a range of sugars (Allerberger 2003; Beumer and Hazeleger 2003). Since selective plating does not distinguish between species of the genus *Listeria*, hemolytic activity (lysing of red blood cells) is the marker used to distinguish *L. monocytogenes* from *L. innocua*, which tends to outgrow and mimic the appearance of *L. monocytogenes* on culture media. Of the six species, only *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* are hemolytic. Hemolytic activity can be detected on horse or sheep blood containing agar plates; however the API-Listeria test, which includes a 'DIM' test, differentiates *L. monocytogenes* from *L. innocua* based on the presence of acrylamidase without considering hemolytic activity (Allerberger 2003). Acrylamidase is present in *L. innocua* strains but absent in *L. monocytogenes* (Billie *et al*. 1992). The API-Listeria consists of the following ten tests. DIM tests for the presence or absence of acrylamidase, ESC tests for the hydrolysis of esculin, α-MAN tests for the presence of α-mannosidase, DARL tests for acid production from D-arabitol, XYL tests for acid production from D-xylose, RHA tests for acid production from L-rhamnose, MDG tests for acid production from α-methyl-D-glucoside, RIB tests for acid production from D-ribose, G1P tests for acid production from glucose-1-phosphate and TAG tests for acid production from D-tagatose.

## 2.7.2 MOLECULAR BASED DETECTION METHODS

Complementing conventional with molecular methods for epidemiology studies regarding food-borne outbreaks or in surveillance systems is fundamental for the prevention and control of human listeriosis. A generic method for the isolation and sub-typing of *L. monocytogenes* from food using conventional and molecular methods are described in Figure 2.10.

### 2.7.2.1 Detection of microorganisms by the PCR reaction

Polymerase Chain Reaction (PCR) is a technique employed for the rapid detection of microorganisms within food products (Kim *et al*. 2001; Holko *et al*. 2002; Gouws and Liedemann 2005). It is a very specific and sensitive technique, which was discovered by Kary M. Mullis (Mullis 1990). PCR is a molecular based method, known for overcoming the limitations of conventional methods for the detection of microorganisms (Al-Soud 2000). The detection of microorganisms by the PCR reaction is divided into four stages namely, sample collection, sample preparation, amplification and detection (Figure 2.11) (Lantz 1998). As a result of the low concentration of pathogens in some complex food samples as well as the presence of PCR inhibitors that will reduce or block DNA amplification, samples have to be treated (pre-PCR treatment) as to increase bacterial numbers to facilitate detection by PCR thereby enhancing DNA recovery. A limitation of the PCR reaction is that various inhibitors present in biological samples could limit its

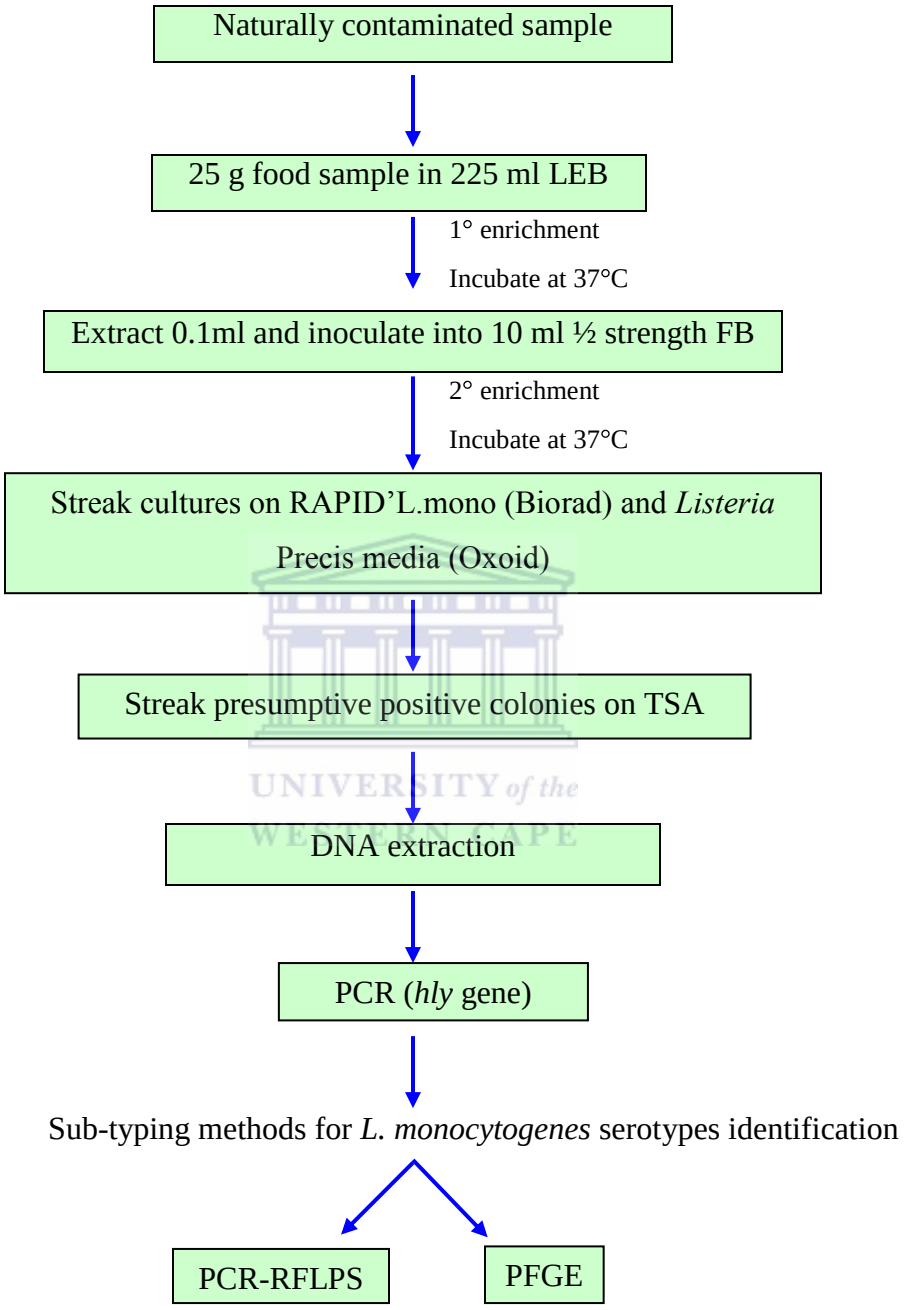



**Figure 2.10** Generic method for the isolation of *L. monocytogenes* in food products and subsequent sub-typing using molecular methods.

potential. These inhibitors tend to interfere with the cell lysis step, inactivate the DNA polymerase or interfere with the nucleic acids rendering a false negative result in the PCR reaction (Al-Soud and Rådström 2000). The sample preparation step is considered to be the most important and necessary step in determining the PCR results since it reduces/eliminates problems associated with PCR inhibitors and determining the sensitivity of the PCR reaction (Lantz 1998). To validate the accuracy of PCR as a powerful molecular tool for pathogen detection, it is important that false-positives or false-negatives be distinguished from true-negative PCR results. This can be achieved by the addition of an internal amplification control (IAC) which is co-amplified with the target DNA under the same conditions. Where the IAC signal is absent, it would indicate that the PCR reaction has failed (Rip and Gouws 2009).



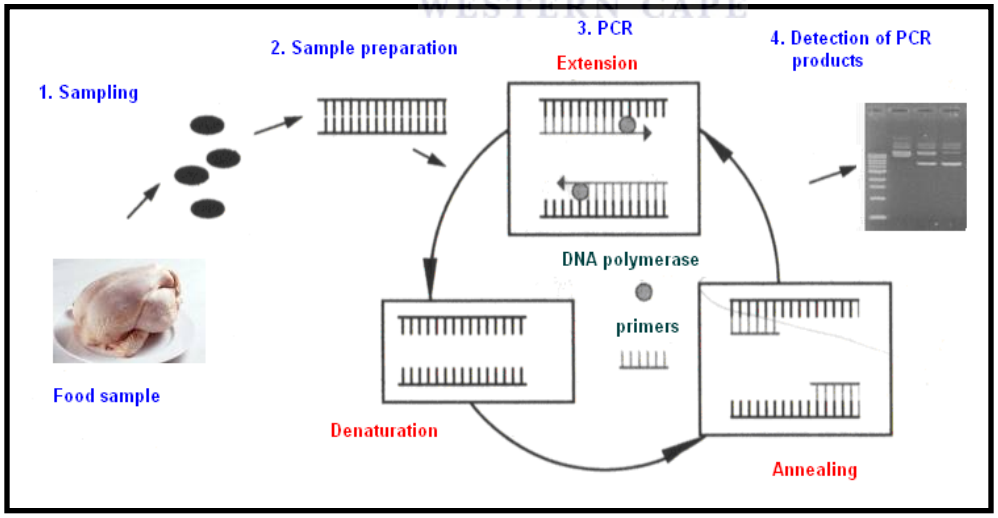



**Figure 2.11** The detection of micro-organisms by PCR. This process is usually divided into 4 stages, namely sample collection, sample preparation, DNA amplification and detection (Adapted from Lantz 1998).

### 2.7.2.2 PCR facilitators

The basic components of a PCR mixture are template DNA, pH buffer, magnesium ions, deoxynucleotides, primers and thermostable DNA polymerase. The amount of template in the reaction strongly influences performance in the PCR reaction. The recommended amount of template for the standard PCR reaction is 1-10 ng for bacterial DNA and 0.1-1 ng for plasmid DNA (Roche PCR Application Manual 1999).

### 2.7.2.3 PCR inhibitors



Specific food components have been shown to inhibit the PCR reaction and the identification of these components can assist when trying to increase the sensitivity of the PCR reaction in order to detect the pathogen present (Kim *et al*. 2000). Certain components have been identified as PCR inhibitors such as hemoglobin, haemin, immunoglobulin G, lactoferrin in blood, myoglobin in muscle, bile salts, complex polysaccharides, proteinases in faeces, anticoagulants, collagen, sodium polyanetholesulfonate (SPS) (a common additive to blood culture medium which tends to co-purify with the DNA), substances in milk and soft cheeses (Akane *et al*. 1994; Fredericks and Relman 1998; Kim *et al*. 2000; Stöcher *et al*. 2003). For this reason, it is therefore necessary to design pre-PCR treatments in order to reduce the effects of PCR inhibitors and thereby maximize DNA recovery when trying to isolate *L. monocytogenes* from food products.

## 2.8 HACCP AS A FOOD SAFETY MEASURE

Food safety is when all conditions in the food chain from production to consumption have been assessed to ensure that no health risks exist when consuming the food product. The safety of the food is compromised when these conditions have not been met (Beumer and Hazeleger 2003). Hazard Analysis and Critical Control Point (HACCP) is a system that is currently being used in the food industry by the government regulatory agencies because it has shown to be effective in preventing the occurrence of food-borne biological, chemical and physical hazards. It is essentially a system of control, which is based on the prevention of problems. HACCP looks for hazards, or what could go wrong, to make a product unsafe for human consumption. Thereafter, control and management systems are implemented to ensure that the product is safe and cannot cause harm to the consumer (Mortimore and Wallace 1994). The implementation of HACCP with effective critical control points to control or prevent *L. monocytogenes* contamination in the food-processing environment has been regarded as an effective measure to control or prevent listeriosis outbreaks and should be implemented by food producers and food preparers (Choi and Hong 2003; Beumer and Hazeleger 2003; Jemmi and Stephan 2006; Curtis 2007).



Where raw milk or pasteurized milk is used to manufacture cheese, it is vital to look at the whole process so that critical control points (CCP) can be identified (Leite *et al*. 2006). The HACCP system to control the presence of *L. monocytogenes* in pasteurized

milk focuses on the selection of raw milk as well as controlling the processing, packaging, distribution and storage conditions (Xanthiakos *et al*. 2006). Since *L. monocytogenes* is commonly isolated from products in the fish-processing environment such as cold smoked fish, HACCP programmes are vital for the seafood industry (Norton *et al*. 2001). The spread of bacteria to food products should be prevented in the food processing environment by implementing HACCP as a system of control. The critical points need to be monitored regularly to prevent *L. monocytogenes* contamination (Rørvik 2000).



The HACCP system was originally developed as a safety system for manned space programmes in the United States to ensure food safety for the astronauts, but it was discovered that a high level of food safety assurance was required, which resulted in an effective HACCP system being implemented (Mortimore and Wallace 1994).

### 2.8.1 The seven principles of HACCP

These include conducting a hazard analysis, identifying the CCP, establishing critical limits, CCP monitoring requirements, corrective actions, record keeping and verification procedures. These HACCP principles have international acceptance. They outline how to establish, implement and maintain a HACCP plan for the system under investigation (National Advisory Committee on microbiological criteria for foods 1997).

## 2.9 CONCLUSION

*Listeria monocytogenes* is a serious threat to the safety of food and its presence in food is an indicator of poor hygiene. Since certain regulatory agencies have issued a zero tolerance ruling for the presence of *L. monocytogenes* in certain food products, effective detection, isolation and confirmation methods are crucial. The ability to differentiate between *L. monocytogenes* strains is important from an epidemiological viewpoint and the tracking of environmental contamination. Sub-typing methods have to be reliable, robust and reproducible since it is a crucial factor in the study of outbreaks of listeriosis. Surveillance has also become increasingly important due to an increase in international trade in foods.



Since the recognition of *L. monocytogenes* as a pathogen causing listeriosis, there has been an advance in the development of methods suitable for isolation and detection. A limitation that may arise when using selective media for *L. monocytogenes* detection is that differentiation between the species is not always possible. Therefore, more advanced, rapid methods such as PCR is employed which is able to differentiate at the species level. By optimizing the PCR protocol, improvements in the quality control of food products will result as well as an increase in knowledge on these pathogens and in which food products they prevail. The use of sub-typing methods such as PFGE, allows for the interpretation of data with greater accuracy. This electrophoretic technique allows for better resolution of DNA fragments.

Sub-typing methods that are used to study infectious disease contributes useful information to epidemiological surveillance. Genetic sub-typing techniques including sequence determination of the virulence genes have allowed for the classification of *L. monocytogenes* serotypes into lineage groups with a difference in virulence potential being displayed between lineage groups. Research on the genes encoding serotype-specific antigens would assist in understanding the mechanism behind the regulation of *L. monocytogenes* serotype proteins (Liu 2006).

Food-borne illness has stressed food safety systems resulting in the need for adequate surveillance systems and legislations being implemented for improved consumer protection. Food safety assurance has to be provided due to rapidly increasing global sourcing of raw materials and the distribution of finished products.

# CHAPTER 3

## The suitability of using SNPs in the *plcA* and the *plcB* genes as a serotyping tool for *Listeria monocytogenes*

## 3.1 ABSTRACT

**Aim**: The aim of this study was to determine single nucleotide polymorphisms (SNPs) for all *Listeria monocytogenes* serotypes within a region of the *plcA, plcB* and *iap* genes. Molecular techniques would then be applied to genotype a strain.



**Materials and Methods**: The nucleotide sequences of the *iap*, *plcA* and *plcB* genes were available in the GenBank/NCBI database. The sequences were aligned and analyzed in the BioEdit sequence alignment editor programme. SNPs (nucleotide differences) were identified within regions of all the genes and primers were designed to amplify regions of the *plcA* and *plcB* genes. The Webcutter programme was utilized to determine whether the SNP was part of an enzyme recognition site. Once SNPs were identified that were unique to the *L. monocytogenes* serotypes, RFLP analysis was performed to differentiate between 12 serotypes of *L. monocytogenes*.

**Conclusion**: Bioinformatic techniques were used to identify SNPs within virulent genes of *L. monocytogenes* for possible use as a serotyping tool. Once PCR and sequencing of

the *plcA* and *plcB* gene of *L. monocytogenes* strains was carried out, it was discovered that SNPs were not conserved in strains with the same serotypic designation so RFLP analysis could not be performed because results would not be consistent and conclusive.

**Significance and Impact of study**: This study highlighted that there are variations of SNPs at the same nucleotide positions within strains with the same serotypic designations. This implies that there are limitations to using RFLP analysis as a sub-typing method on certain virulent genes for differentiating between serotypes on the basis that SNPs, thought to be specific for a serotype, are not conserved at certain positions within these genes.

## 3.2 INTRODUCTION

Epidemiology research requires methods that can differentiate between *Listeria monocytogenes* strains. The development of typing methods to trace the strains of *L. monocytogenes* involved in the outbreak of listeriosis would help limit the spread of the disease (Liu *et al*. 2006). These methods provide epidemiological markers, critical for disease outbreak investigations (Nadon *et al*. 2001). The epidemiological data will be useful in determining the pathogenic potential of certain strains over others and in turn would be important to further reduce the occurrence of listeriosis (Liu 2006).



Serotyping was one of the first diagnostic techniques developed for *L. monocytogenes* (Seeliger and Hőhne 1979). Serotyping has identified 13 known serotypes of *L. monocytogenes*. The serotypic designation of the strain is on the basis of a serological reaction between *L. monocytogenes* somatic (O) and flagellar (H) antigens (carried on their surface) with specific antisera (Zhang *et al*. 2003; Rebuffo-Scheer *et al*. 2007; de Vasconcelos *et al*. 2008; Jeyaletchumi *et al*. 2010). Latex agglutination tests are commercially available for the identification of *Listeria monocytogenes* serotypes.

Certain surface antigens may be associated with virulence and pathogenesis since studies have shown that the expression of antigens specific to a serotype may be implicated in the ability of *L. monocytogenes* to invade mammalian cells (Lei *et al*. 1997) hence serotype designation is associated with virulence potential. Of the 13 serotypes of *L.*

*monocytogenes*, it has been reported that 1/2a, 1/2b and 4b are responsible for more than 95% of listeriosis infections in humans (Dussurget *et al*. 2004; de Vasconcelos *et al*. 2008; Jeyaletchumi *et al*. 2010; Ward *et al*. 2010) although the virulent genes are inherent to all serotypes (Zhang *et al*. 2003). *L. monocytogenes* serotypes 1/2a and 1/2b are mainly associated and isolated sporadically from food and 4b is responsible for the major human epidemic cases (Borucki and Call 2003; Gilbreth *et al*. 2005; Smidt *et al*. 2005; Ward *et al*. 2010).

Alternative molecular approaches to serotyping has been explored since serotyping is laborious and delivers poor discriminatory power between strains that are non-typeable (Carminati *et al*. 2004; Gilbreth *et al*. 2005; Bou-m'handi *et al*. 2007; Garrido *et al*. 2008). Although it has always been the typing method of choice, it has its limitations with regard to high costs, the technical expertise required to perform the assays, the availability of reagents and the need for high quality antisera (Borucki and Call 2003; Doumith *et al*. 2004; Kérouanton *et al*. 2010). For epidemiological investigations, alternative sub-typing methods for *L. monocytogenes* serotype identification have been explored to attain greater discrimination of strains and to increase the speed and reproducibility of sub-typing *L. monocytogenes*. Methods include phenotypic sub-typing methods namely, phage-typing, multilocus sequence sub-typing (MSS) and genetic sub-typing methods namely, rapid amplification of polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), microarray genomic analysis, PFGE, ribotyping, serotype specific PCR; including MAMA-PCR, multiplex PCR and RFLP-

PCR (Jinneman and Hill 2001; Borucki and Call 2003; Dhanashree *et al*. 2003; Zhang *et al*. 2003; Doumith *et al*. 2004; Tominaga 2006). Serotyping has been regarded as the most important phenotypic sub-typing method; however phenotyping methods have largely been replaced by genetic sub-typing methods in recent years (van Belkum *et al*. 2007) since it provides more sensitive strain discrimination and reproducibility (Wiedmann 2002; Revazishvili *et al*. 2004).

Almost full length invasion associated proteins (*iap*) gene sequences were established for all 13 serotypes of *L. monocytogenes* in the GenBank/NCBI database. The gene sequences of the *plcA* gene were also determined for all 13 serotypes of *L. monocytogenes* and the *plcB* gene for 10 of the 13 isolates. Partial sequences of these virulent genes were sufficient to characterize the *L. monocytogenes* serotypes into lineage groups (Schmid *et al*. 2003). A comparison of these *L. monocytogenes* sequences grouped the strains into 3 genotypes/lineages.



Along with other *L. monocytogenes* virulent genes, the *plcA* and *plcB* genes act as virulent factors in the invasion process of the host cell. Listeria secretes two Phospholipase Cs (PLCs) involved in the lysis and escape from intracellular vacuoles. Phospatidylinositol (PI)-PLC, encoded by the *plcA* gene, aids in the escape of *L. monocytogenes* from the host's primary vacuole while phosphatidylcholine (PC)-PLC is involved in the cell-to-cell spread of the bacteria. PC-PLC encoded by the *plcB* gene, acts with listeriolysin O (LLO – a protein encoded by the *hly* gene) to lyse its host's primary

and secondary vacuoles. Once in the cytoplasm, the organism will acquire nutrients from the cytosol and multiply (Vázquez-Boland *et al*. 2001a; Dussurget *et al*. 2004; Schmid *et al*. 2005).

It was demonstrated that *L. monocytogenes* with mutations in the genes coding for the enzymes PI-PLC and PC-PLC are less virulent to mice than wild-type bacteria and that PC-PLC can be a substitute for LLO with regard to virulence since it can damage vacuolar membranes in epithelial cells (Doyle 2001). In a study undertaken by Témoin *et al*. (2008), it was demonstrated that mutations in the *plcA* gene explains the lack of PI-PLC activity in low virulent *L. monocytogenes* strains.



The *iap* gene of *L. monocytogenes* encodes an extracellular protein p60 which plays a role in the invasion and adherence of this organism to cells (Bubert *et al*. 1999). In all *Listeria* spp. it acts as a murein hydrolase necessary for proper cell division. The *iap* sequences have conserved N- and C- terminal domains flanking a variable region which contains a number of TN repeats (Schmid *et al*. 2005).

In order to differentiate between the serotypes of *L. monocytogenes* in the *plcA*, *plcB* and *iap* genes, the approach was to first look at SNPs within selected regions of these genes. Polymorphisms result as a consequence of a mutation. There are a number of polymorphisms which can be defined by the type of mutation that caused it. The simplest type of polymorphism, namely SNP, results from a mutation in a single base which is

then substituted by another nucleotide. SNPs refer to a change in a single nucleotide that can occur in a DNA sequence (Schork *et al*. 2000). For example, when comparing a conserved region in a gene in a number of serotypes and there is a variation in a single nucleotide where nucleotide guanine (G) replaces one of the other 3 nucleotides, namely; cytosine (C), adenine (A), or thymine (T), then G is considered the SNP. SNPs are among the most common genetic variations and may also serve as biological markers to identify genes associated with disease (Sherry *et al*. 2001). SNPs may affect the functioning of a gene and its ability to promote illness/disease. However, due to the degeneracy of the genetic code, SNPs may not alter the amino acid sequence of a protein and as a result have no effect on the functioning of a gene. SNPs occur every 100 to 300 bases along the 3 billion base human genome and two of every three SNPs involve the replacement of C with T (Human Genome Project Information 2008). The digestion of a piece of DNA, containing the SNP, with the relevant restriction enzyme could distinguish alleles or variants as a result of the fragment sizes generated via gel electrophoresis. This type of polymorphism is termed RFLPs (Schork *et al*. 2000). *L. monocytogenes* strains involved in invasive and noninvasive listeriosis outbreaks have been characterized by DNA typing methods and the differences detected among their DNA sequences suggests that there may be a difference in the expression of various virulence factors. For this reason, it is suggestive that some strains in the environment or food may be more virulent over others and not pose the same threat to human health (Doyle 2001). Virulent genes are inherent to all the serotypes of *L. monocytogenes*, however some strains are avirulent or weakly virulent which suggests that virulence is attributed to the levels of expression of virulence

factors, partially due to sequence variations in their virulence genes (Doyle 2001; Chen *et al*. 2005).

The aim of this study was to determine SNPs within a region of the *plcA, plcB* and *iap* genes for all the *L. monocytogenes* serotypes and to determine the suitability of using PCR-RFLPs for the chosen genes. This would allow for the differentiation between serotypes in order to advance with molecular techniques to determine which serotypes are more prevalent over others in causing food-borne illness and human listeriosis.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Bioinformatics tools to determine SNPs within the virulent genes

The nucleotide sequence of the *plcA*, *plcB* and *iap* genes for all serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4ab, 4d, 4e, 7) were downloaded from the Pubmed NCBI database. These genes were selected since the nucleotide sequences of the 13 serotypes were available from the GenBank/NCBI database (Table 3.1) (Schmid *et al.* 2003). The Bioedit programme was selected and used to align and analyze all the 13 serotypes for the three respective genes. Within this programme, SNPs were identified for each serotype of a gene. Once the SNP was identified, the Webcutter 2.0 programme (http://bio.lundberg.gu.se/cutter2/) was used to determine whether an enzyme cuts at a region including the SNP. Where the SNP of a serotype is not part of an enzyme recognition site of one gene e.g. the *plcA* gene, SNPs were then looked for in the *plcB* and *iap* genes. Where no enzyme cuts at the SNPs for a serotype within any of the three genes, an alternative procedure was adopted whereby the serotype would be left uncut (indicating a positive result) opposed to the remainder of the 12 serotypes being cut by one enzyme at that same position within the gene (depending on whether an enzyme recognition site was present in the other serotypes). The serotype of choice is left uncut while the remaining serotypes are cut by the enzyme generating the same digest profile for 12 of the 13 serotypes (this method, however, was not required or utilized for this research). Once a SNP that was part of an enzyme recognition site was identified for all

serotypes, PCR was carried out using primers specific for each gene. Primers were designed for the *plcA* and *plcB* genes using the Oligoanalyzer 3.0. programme (Table 3.2). In the *iap* gene, there was only one of the 13 serotypes with a SNP part of an enzyme recognition site and as a result the approach was to first determine whether the PCR-RFLP approach was successful in the *plcA* and *plcB* genes before designing primers for the *iap* gene. When selecting the SNP, it was taken into account that the SNP, if allowed, had to be far enough away from the primer region (approximately 100 bp) to ensure that once the RFLP technique was performed, the digested DNA was large enough to be easily viewed after gel electrophoresis, especially where 2 fragment sizes differed remarkably in size.



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**Table 3.1** A list of the sequenced *L. monocytogenes* strains used as a reference from the GenBank/NCBI database (Schmid *et al*. 2003)

| ***L. monocytogenes* serotype** | **Culture collection** | **GenBank accession numbers of the *plcA* gene** | **GenBank accession numbers of the *plcB* genes** | **GenBank accession numbers of the *iap* genes** |
|---|---|---|---|---|
| 1/2a | TUMLis18 | AL591974 | X559723 | X52268 |
| 1/2b | SLCC 2755 | AF532205 | AY150836 | AF532277 |
| 1/2c | NCTC 5348 | AF532206 | AY150830 | AF532254 |
| 3a | NCTC 5105 | AF532207 | AY150831 | AF532259 |
| 3b | SLCC 5543 | AF532216 | AY150837 | AF532261 |
| 3c | SLCC 2479 | AF532208 | - | AF532258 |
| 4a | SLCC 2374 | AF532211 | AY150841 | AF532229 |
| 4ab | SLCC 4561 | AF532210 | - | AF532257 |
| 4b | SLCC 4013 | AF532211 | AY150833 | AF532287 |
| 4c | ATCC 19116 | AF532212 | AY150832 | AF532227 |
| 4d | ATCC 19117 | AF532213 | AY150834 | AF532291 |
| 4e | ATCC 19118 | AF532214 | - | AF532293 |
| 7 | SLCC 2482 | AF532215 | AY150835 | AF532276 |

ATCC – American Type Culture Collection; NCTC – National Collection of Type Culture; SLCC – Special Listeria Culture Collection; TUM – Technical University of Munich, Institute of Microbiology.

**Table 3.2** The primers that were designed to amplify regions of the *plcA* and *plcB* gene for serotype identification

| Primer | Sequence | Melting temperature (Tm) | GC content | Amplicon size |
|---|---|---|---|---|
| Forward: plcA | 5' CCA GGT ACA CAT GAT ACG ATG 3' | 52.7°C | 47.6% | 814 bp |
| Reverse: plcA | 5' CCG AGG TTG CTC GAG ATA TAC 3' | 54.6°C | 52.3% | |
| Forward: plcB | 5' GTG GTT CTA GGT ATG TGC 3' | 49.8°C | 50% | 701 bp |
| Reverse: plcB | 5' CGG GTA GTC CGC TTT CGC C 3' | 61.1°C | 68.4% | |

### 3.3.2 Reference strains



*Listeria monocytogenes* reference strains 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 7 were obtained from the Pasteur Institute in France. Nottingham University provided serotypes 1/2a, 1/2b, 1/2c and 4b for this research and the University of the Western Cape had serotype 1/2a and 4b in their culture collection (serotype reference numbers available in Table 3.3). Serotype 4ab was the only serotype not obtained due to its scarcity. After searching on the world culture collection database (http://wdcm.nig.ac.jp/hpcc.html), it was found that there was no referenced serotype 4ab strain available for purchasing.

### 3.3.3 Reference cultures

Freeze-dried cultures of the *L. monocytogenes* serotypes obtained from the Pasteur Institute in France were re-cultured according to their instructions. Briefly, they were resuspended in 200 µl tryptone soy broth (TSB) (Oxoid) and 20 µl of this suspension was inoculated into 10 ml TSB (Oxoid) and incubated overnight at 37°C. *L. monocytogenes* strains obtained from Nottingham University were sent on a swab in a vial. Bacteria on the swab were immersed in 10 ml TSB (Oxoid) and incubated overnight in a shaker (114 rpm) at 37°C.


### 3.3.4 DNA isolation from reference cultures

A method modified by Agersborg *et al*. (1997) was used for DNA isolation. Following the overnight enrichment, a 2.0 ml aliquot culture was transferred to an eppendorf tube. Cultures were centrifuged at 9000 x g for 10 min. The pellet was resuspended in 400 µl sterile distilled water to which 400 µl 2 % Triton-X-100 (BDH Chemicals Ltd) was added and the contents mixed for 5 s. This suspension was left at room temperature for 10 min, thereafter incubated at 100°C for 10 min and centrifuged at 9000 x g for 4 min. The supernatant was transferred to a sterile eppendorf tube and 1 µl of this crude cell lysate was used for PCR amplification.

### 3.3.5 Primer design for the *plcA* and *plcB* gene

Primers were designed to amplify full length sequenced gene products for the *plcA* and *plcB* genes resulting in a PCR product of approximately 814 bp and 701 bp respectively. The primer sequences were designed using the OligoAnalyzer 3.0 and BLAST-n (Basic Local Alignment Search Tool) was used to ensure that the selected oligonucleotide primers would not recognize and anneal to any other sequence but that of the *plcA* and *plcB* gene sequences. The Tm and % GC content of the primer sets are listed in Table 3.2.


### 3.3.6 Gradient PCR

To determine the optimal annealing temperature for primer sets, gradient PCR was employed using the Eppendorf Mastercycler gradient (Merck). Temperature ranges of 49°-57°C were used to determine optimal annealing temperatures for primer sets of the *plcA* and *plcB* genes. Optimal annealing temperatures were observed at 55°C for the amplification of the *plcA* gene and 57°C for the *plcB* gene.

### 3.3.7 PCR amplification

For a 25 µl reaction, the mixture contained: 1 X PCR buffer (final concentration) (Bioline), $MgCl_2$ (final concentration 5 mM) (Bioline), dNTP's (final concentration 200 µM) (Roche Diagnostics), primers plcA (F), plcA (R), plcB (F) and plcB (R) (final concentration 0.3 µM each) (Whitehead Scientific), 1U *Biotaq* DNA polymerase (Bioline), 1 µl crude extract ($10^0$).

Amplification was carried out in a thermal cycler GeneAmp® PCR system 2700 (Applied Biosystems) with the following programme: Initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for the *plcA* gene and 57°C for the *plcB* gene for 40 s, extension at 72°C for 40 s and a final extension step at 72°C for 5 min. The PCR products underwent electrophoresis on a 1.5% agarose D-1 LE gel (Whitehead Scientific) and visualized by staining with ethidium bromide. The amplified PCR products were viewed using the Alphaimager® HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC™ software version 4.0.0.

### 3.3.8 RFLP analysis

RFLP analysis was carried out separately for all serotypes with the respective enzymes (Table 3.3). For a 10 μl restriction digest, the mixture contained; 5 μl PCR product, 1 X restriction buffer (final concentration) (Fermentas and New England Biolabs) and restriction enzyme (final concentration 1U/μl) (Fermentas and New England Biolabs). The DNA was digested at 37°C for 16 h and the reaction inactivated at different temperatures as described by the manufacturer. The restriction digest products underwent electrophoresis on a 1.5% agarose D-1 LE gel (Whitehead Scientific) and visualized by staining with ethidium bromide. An uncut DNA sample was used as a negative control. The digested DNA was viewed using the Alphaimager® HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC™ software version 4.0.0.



The results obtained from RFLP analysis showed that the digest did not work and that either, the DNA remained uncut or undesired / non-specific banding patterns were produced. Following this negative result, various enzyme concentrations were tested (concentrations up to 5U/μl) (data not shown). Results still showed negative for the restriction digest.

**Table 3.3** The enzymes used to perform RFLP analysis where a SNP was present in a specific gene

| Gene | Serotype | Reference no | SNP * | Enzyme | Cut site | Other serotype** | Digest pattern (bp) |
|---|---|---|---|---|---|---|---|
| *plcA* | 1/2c | CIP 105.448 / LM 0048-0305 | T624A | *Alu*I | AG/CT | 3c | 41, 75, 189, 486 |
| | 3b | CIP 78.35 | A719G | *Bcn*I | CC/SGG | - | 98, 716 |
| | 3c | CIP 78.36 | A653G | *Hin*fI | G/ANTC | - | 161, 653 |
| | 4b | ATCC 23074 / ScottA (UWC L1) | A60C | *Van*9II | CCANNNN/NTGG | - | 60, 754 |
| | 4d | CIP 105.458 | G562T | *Rsa*I | GT/AC | - | 27, 88, 97, 155, 447 |
| | 4e | CIP 105.459 | T461C | *Hpa*II | C/CGG | - | 353, 461 |
| | 7 | CIP 78.43 | N/A | | | - | |
| *plcB* | 1/2a | LM 1040-35 NCTC 7973 (UWC L3) | A698G | *Hpy*F10VI | GCNNNNN/NNGC | - | 20, 35, 182, 464 |
| | 1/2b | CIP 105.448 / LM 0054-0305 | A298G | *Fok*I | GGATG | - | 252, 449 |
| | 3a | CIP 78.35 | T540A | *Ssp*I | AAT/ATT | - | 209, 492 |
| | 4a | CIP 105.457 | G602C | *Cac*8I† | GCN/NGC | - | 33, 148, 235, 285, |
| | 4c | CIP 54.135 | A440G | *Bfa*I† | C/TAG | - | 27, 66, 71, 219, 292 |

* One approach to write SNPs is the nucleotide followed by the SNP location and base substitution e.g A719G refers to nucleotide A being substituted by G at the nucleotide position 719 in the gene

N/A – no SNPs were part of an enzyme recognition site therefore RFLP analysis could not be carried out for *L. monocytogenes* serotype 7 of the *plcA* and *plcB* genes

** other serotypes that shares the SNP at the same nucleotide position

† Only these enzymes were supplied by New England Biolabs otherwise Fermentas was the supplier

### 3.3.9 Sequencing

The PCR products of the DNA amplified for all the *L. monocytogenes* serotypes were sent for purification and sequencing at the Stellenbosch DNA sequencing unit at Stellenbosch University in order to determine why the expected digest profiles were not obtained. Either the serotyping of the strains needed to be redone or it had to be determined whether the SNP was indeed present within the *L. monocytogenes* serotypes of the *plcA* and *plcB* genes.

## 3.4 RESULTS AND DISCUSSION

A region of the *plcA* and *plcB* gene of *L. monocytogenes* was amplified by PCR using the primers that were designed for the amplification of a 814 bp and 701 bp region respectively (Figure 3.1 and Figure 3.2). Once the PCR products were subjected to sequencing, they were analyzed by importing the nucleotide sequences into the BioEdit programme. The nucleotide sequences of the *L. monocytogenes* serotypes were aligned (for the *plcA* and *plcB* genes) and it was discovered that the SNPs of the *L. monocytogenes* serotypes were in fact not in the same place as those present in the sequences downloaded from the NCBI database (Table 3.1) (Schmid *et al.* 2003). The DNA sequences for all the strains of the *plcA*, *plcB* and *iap* genes can be found in the GenBank database under the accession numbers listed in Table 3.1. This information showed that there is variation in the SNPs between isolates of the same serotypes within the *plcA* and *plcB* genes and for this reason RFLP analysis for the differentiation between the *L. monocytogenes* serotypes would not work (Figure 3.3). The expected digest profile / band sizes are listed in Table 3.3. The serotypes of *L. monocytogenes* that remained uncut included serotypes 1/2b, 3b, 3c, 4b and 4e that should have been digested by the enzymes selected had the SNP been present like it appears in the sequenced strains in the GenBank/NCBI database (Schmid *et al*. 2003). For example, a comparison between the sequenced *plcA* gene products for serotypes 1/2c from the Pasteur Institute in France (CIP 105.448), Nottingham University (LM 0048-0305) and the reference strain (NCTC 5348) from the NCBI database, it was apparent that the SNPs were not conserved but a

variation of SNPs between isolates of this same serotype (at the same nucleotide positions) had occurred. This was also the case for the other serotypes.

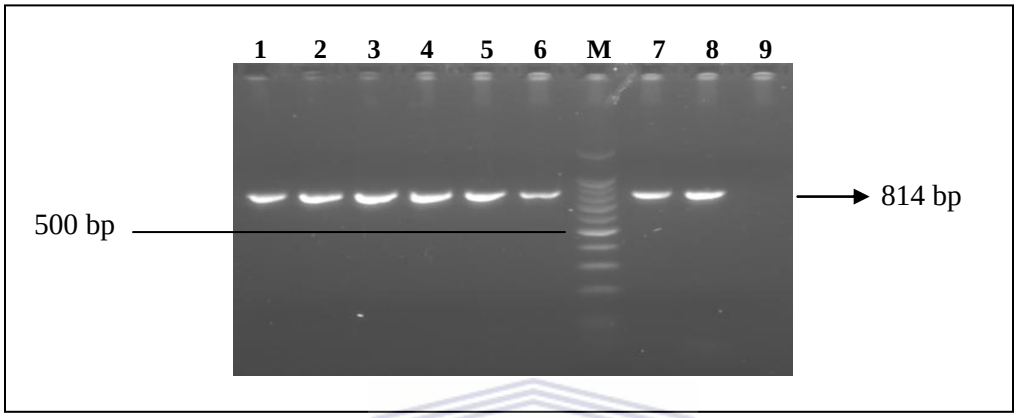


**Figure 3.1** Gradient PCR for the amplification of an 814 bp region of the *plcA* gene of *L. monocytogenes* serotype 4b (UWC L2). **Lanes 1-9**: annealing temperatures 49°-56°C were used. **M**: 100 bp DNA ladder (Promega); **lane 1**: 56°C; **lane 2**: 55.5°C; **lane 3**: 54.6°C; **lane 4**: 53.3°C; **lane 5**: 51.8°C; **lane 6**: 50.5°C; **lane 7**: 49.5°C; **lane 8**: 49°C; **lane 9**: negative control (water).

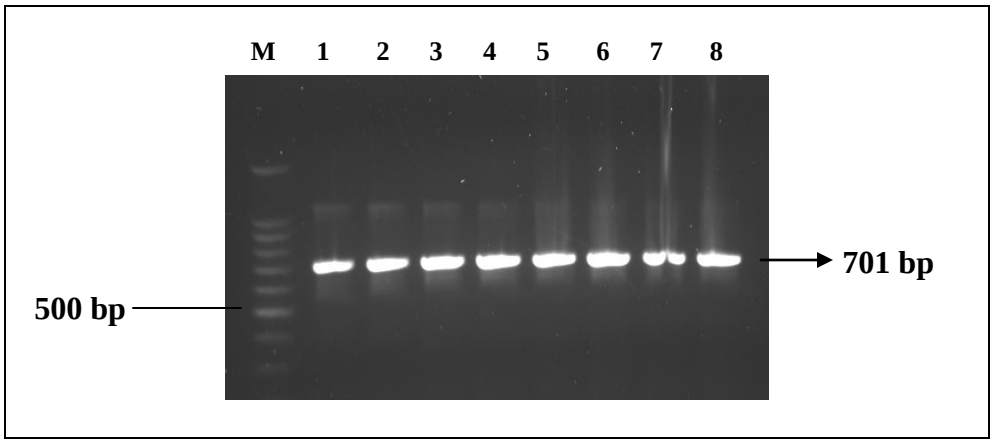


**Figure 3.2** Gradient PCR for the amplification of a 701 bp region of the *plcB* gene of *L. monocytogenes* serotype 1/2c (CIP 105.448). **M**: 100 bp DNA ladder (Promega); **lanes 1-8**: annealing temperatures 49°-57°C were used. **lane 1**: 57°C; **lane 2**: 56.5°C; **lane 3**: 55.4°C; **lane 4**: 54°C; **lane 5**: 52.2°C; **lane 6**: 50.7°C; **lane 7**: 49.6°C; **lane 8**: 49°C.

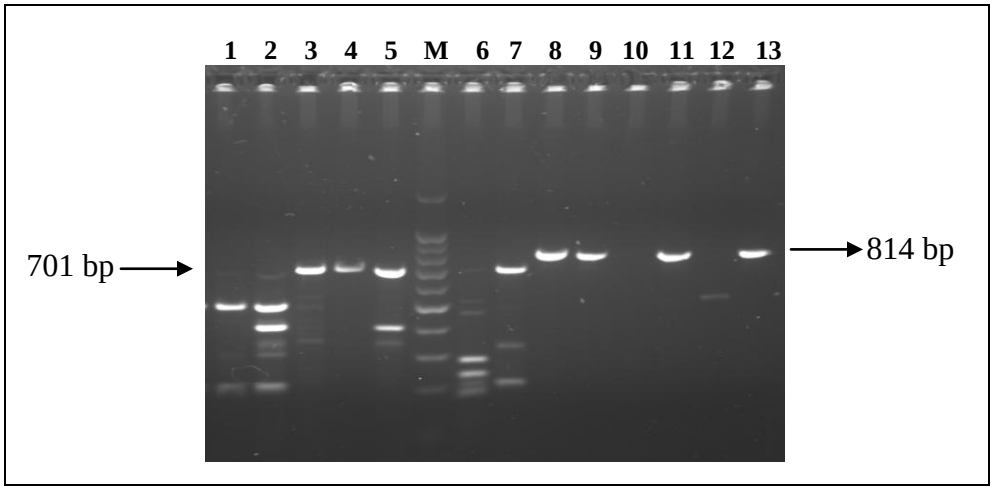


**Figure 3.3** RFLP analysis of PCR amplified DNA of *L. monocytogenes* serotypes 3b, 3c, 4b, 4d, 4e of the *plcA* gene and 1/2a, 1/2b, 3a, 4a and 4c of the *plcB* gene. **M**: 100 bp DNA ladder (Promega); **lanes 1-5, 6, 7**: a 701 bp amplicon of the *plcB* gene that underwent RFLP analysis; **lane 1**: *L. monocytogenes* serotype 1/2a (UWC L3) digested with enzyme *Hpy*F10VI (Fermentas); **lane 2**: *L. monocytogenes* serotype 1/2a (LM 104035) digested with enzyme *Hpy*F10VI (Fermentas); **lane 3**: *L. monocytogenes* serotype 1/2b (CIP 105 449) digested with enzyme *Fok*I (Fermentas); **lane 4**: *L. monocytogenes* serotype 1/2b (LM 0054 0305) digested with enzyme *Fok*I (Fermentas); **lane 5**: *L. monocytogenes* serotype 3a (CIP 78.34) digested with enzyme *Ssp*I (Fermentas); **lane 6**: *L. monocytogenes* serotype 4a (CIP 105 457) digested with enzyme *Cac*8I (Fermentas); **lane 7**: *L. monocytogenes* serotype 4c (CIP 54.135) digested with enzyme *Bfa*I (Fermentas); **lanes 8-13**: a 814 bp amplicon of the *plcA* gene that underwent RFLP analysis; **lane 8**: *L. monocytogenes* serotype 3b (CIP 78.35) digested with enzyme *Bcn*I (Fermentas); **lane 9**: *L. monocytogenes* serotype 3c (CIP 78.36) digested with enzyme *Hinf*I (Fermentas); **lane 10**: *L. monocytogenes* serotype 4b (UWC L1) digested with enzyme *Van*9II (Fermentas); **lane 11**: *L. monocytogenes* serotype 4b (ATCC 23074) digested with enzyme *Van*9II (Fermentas); **lane 12**: *L. monocytogenes* serotype 4d (CIP 105 458) digested with enzyme *Rsa*I (Fermentas); **lane 13**: *L. monocytogenes* serotype 4e (CIP 105 459) digested with enzyme *Hpa*II (Fermentas).

Of the *L. monocytogenes* serotypes that displayed a restriction digest profile, namely serotypes 1/2a, 3a, 4a, 4c and 4d, none produced the expected banding profiles. Comparisons between sequenced gene products (different isolates with the same serotypic designations) had confirmed differences between SNPs at the same nucleotide positions. This would account for the differences in the banding profiles obtained compared to that which was expected.

As a result of these inconsistencies, another virulent gene of *L. monocytogenes* had to be selected in order to determine whether there was a region in the gene where SNPs were conserved that could be used for RFLP analysis and in turn to differentiate between the serotypes of *L. monocytogenes*. Once it was confirmed that there were variation of SNPs within the *plcA* and *plcB* genes of *L. monocytogenes*, the *hly* gene of *L. monocytogenes* was selected (a virulent gene that encodes the protein listeriolysin O) for further research.

## 3.5 CONCLUSION

*Listeria monocytogenes* is an opportunistic pathogen that can survive under extreme conditions such as low pH, low temperature and high salt concentrations. These factors, together with the fact that its presence is ubiquitous in nature, allow it to persist in foodstuffs such as raw produce or RTE food products. Of the 13 serotypes; 1/2a, 1/2b and 4b are mainly associated with human listeriosis indicating its virulence potential over other strains. The value of molecular methods in distinguishing between the serotypes of *L. monocytogenes* in epidemiological investigations is important since it may assist with understanding the pathogenic properties of the *L. monocytogenes* serotypes and may help limit the spread of listeriosis.



The results obtained with serotyping may be discrepant since it is dependent on the phenotypic characteristics of the bacteria and as a result, alternative molecular techniques, which are more specific and sensitive, are utilized for the identification and differentiation between *L. monocytogenes* serotypes. In this study, the PCR-RFLP technique was selected for distinguishing between the 12 serotypes of *L. monocytogenes* in the *plcA* and *plcB* genes. The distinction between serotypes was on the basis of SNPs that differed between serotypes. It was discovered that SNPs are not conserved within strains of the same serotype but that SNP variations do occur between strains in the *plcA* and *plcB* gene. The variation of SNPs within strains of the same serotypes resulted in the loss of the restriction site. As a result of these findings, another virulent gene had to be

selected to determine whether SNPs were conserved in a region whereby RFLP analysis could be performed in order to apply a molecular technique which ultimately would provide a sensitive, discriminatory and reproducible method for trace-back and epidemiological investigations of *L. monocytogenes*.

# CHAPTER 4

## A SNP-based PCR assay to differentiate between *Listeria monocytogenes* lineages derived from bioinformatics analysis of the *hly* gene

### 4.1 ABSTRACT

**Aim:** The aim of this study was to determine single nucleotide polymorphisms (SNPs) for all the serotypes of *L. monocytogenes* within a region of the virulent *hly* gene. A PCR-RFLP technique would be developed to rapidly characterize *L. monocytogenes* isolates into lineage groups and this technique would be confirmed by mismatch amplification mutation assay (MAMA)-PCR.



**Materials and Methods:** PCR amplified fragments of the *hly* gene for all 12 serotypes of *L. monocytogenes* were sequenced, aligned and analyzed in the BioEdit programme. SNPs were identified within regions of the 12 serotypes of the *hly* gene. The Webcutter programme was utilized to determine whether the SNP was part of an enzyme recognition site. PCR-RFLP analysis of the *hly* gene was employed to differentiate between lineage groups of *L. monocytogenes*. Mismatch amplification mutation assay (MAMA)-PCR was a technique employed to ensure the results obtained with PCR-RFLP analysis were conclusive.

**Conclusion:** Phylogenetic analysis of the 13 recognized serotypes of *L. monocyogenes* have classified them into lineage groups/genotypes. The *hly* gene of *L. monocytogenes* has a distinct, well conserved region with some variation in the central domain for the differentiation between three lineage groups. In this study, RFLP analysis was carried out to differentiate between lineage groups based on the sequence differences/SNPs within each lineage group. The ability of *L. monocytogenes* to cause disease was shown to have some correlation to the serotypes of a specific lineage group, making distinction between lineage groups important for epidemiological analysis.



**Impact and significance of study:** PCR-RFLP analysis applied in this study placed *L. monocytogenes* serotypes into three lineage groups. Where there was variation of SNPs of isolates of the same serotypes in the *plcA* and *plcB* genes, sequencing of isolates of the same serotype within the *hly* gene showed that SNPs were conserved in a region of this gene. This method of lineage group classification was conclusive since the SNPs were conserved to a region where RFLP analysis could be applied for distinction between *L. monocytogenes* lineage groups.

## 4.2 INTRODUCTION

Phylogenetic analysis of the 13 recognized serotypes of *L. monocyogenes* have classified them into lineage groups/genotypes (Zhang *et al*. 2003; Ward *et al*. 2010) on the basis of partial sequences of some *L. monocytogenes* functional and virulence genes e.g. *iap*, *hly*, *flaA*, *actA* (Schmid *et al*. 2003). There are nucleotide sequence differences between *L. monocytogenes* serotypes within several virulent genes (Jinneman and Hill 2001). Numerous molecular sub-typing techniques have classified *L. monocytogenes* strains into three lineage group. Lineage/genotype I consists of serotypes belonging to flagellar antigen type b and d namely; 1/2b, 3b, 4b, 4d and 4e and lineage II consists of serotypes belonging to flagellar antigen type a or c namely; 1/2a, 1/2c, 3a or 3c except serotypes 4a and 4c (Tran and Kathariou 2002; Borucki and Call 2003; Schmid *et al*. 2003; Borucki *et al*. 2004; Ragon *et al*. 2008). Serotypes 4a and 4c, which are rather uncommon, have been characterized as lineage III isolates due to its genetic distance from other serotypes and that it is rarely implicated in listeriosis but mainly associated with animal infections (Zhang *et al*. 2003; den Bakker *et al*. 2008). It therefore has a lower pathogenic potential in humans.



The ability of *L. monocytogenes* to cause disease varies between lineage groups due to the difference in pathogenic potential of the serotypes (Ward *et al*. 2004). It is then important to be able to distinguish between these lineage groups for epidemiological analysis and to determine the serotypes involved in listeriosis.

The *hly* gene of *L. monocytogenes* has an important role in intracellular parasitism only in this species. This is a result of the production of listeriolysin O (LLO), a protein encoded by the *hly* gene that has hemolytic activity (Seveau *et al*. 2007; Fagundes de Mello *et al*. 2008). A number of factors have been shown to be associated with the virulence of *L. monocytogenes*, one of which is LLO. The *hly* gene of *L. monocytogenes* has a distinct, well conserved region. There is some variation in the central domain of this gene to allow for the differentiation between 3 lineage groups. The fact that there are a small number of sequence differences within each lineage group is indicative of a strong degree of conservation in each lineage (Rasmussen *et al*. 1995).

Restriction endonucleases have proved to be a valuable tool in microbial epidemiology (Farber and Peterkin 1991; Brett *et al*. 1998; Mammina *et al*. 2009). A molecular method based on RFLPs of PCR amplified fragments of the *hly* gene was designed to rapidly distinguish between *L. monocytogenes* lineage groups. This method was optimized using the reference strains of *L. monocytogenes* serotypes. The approach was to determine conserved SNPs within the *hly* gene and then proceed with PCR-RFLP analysis in order to differentiate between *L. monocytogenes* lineage groups. This SNP-based sub-typing scheme was designed to look at the distribution of *L. monocytogenes* serotypes in the food processing environment and those involved in human clinical cases. A more efficient alternative for DNA sequence based sub-typing is represented by the examination of SNPs (Ducey *et al*. 2007).

Mismatch amplification mutation assay (MAMA)-PCR was another molecular tool employed for lineage group classification. This technique was to confirm the method of lineage group classification by RFLP analysis designed and employed in this study. All the *L. monocytogenes* reference strains were included in order to determine the accuracy of this method. This technique works on the principle of detecting single nucleotide changes within genes which can be applied for epidemiological studies. It functions on the basis of using one conserved primer and one mutation detection primer (Alonso *et al*. 2005) to produce a PCR product with a band size specific for a single lineage group. For the mutation/mismatch primer, the SNP which distinguishes between lineage groups, is included in the 3' end of the primer sequence. This MAMA-PCR method is a simple and rapid method for the differentiation between *L. monocytogenes* lineage groups based on single nucleotide differences that exist between lineage groups.

## 4.3 MATERIALS AND METHODS

Since the nucleotide sequences for all the serotypes of the *hly* gene were not available on the GenBank/NCBI database together with the fact that there were differences between NCBI and sequence-verified genes, all the isolates obtained from the Pasteur Institute in France, Nottingham University and UWC were subjected to a DNA isolation procedure, PCR amplification of a 730 bp region of the *hly* gene (Annexure 1) and sequencing.

### 4.3.1 DNA isolation from reference cultures



A method modified by Agersborg *et al*. (1997) was used for DNA isolation. Following the overnight enrichment, a 2.0 ml aliquot culture was transferred to an eppendorf tube. Cultures were centrifuged at 9000 x g for 10 min. The pellet was resuspended in 400 µl sterile distilled water to which 400 µl 2 % Triton-X-100 (BDH Chemicals Ltd) was added and the contents mixed for 5 s. This suspension was left at room temperature for 10 min, thereafter incubated at 100°C for 10 min and centrifuged at 9000 x g for 4 min. The supernatant was transferred to a sterile eppendorf tube and 1 µl of this crude cell lysate was used for PCR amplification.

### 4.3.2 PCR amplification

For a 25 µl reaction, the mixture contained: 1 X PCR buffer (final concentration) (Bioline), $MgCl_2$ (final concentration 5 mM) (Bioline), dNTP's (final concentration 200 µM) (Roche Dagnostics), primers hlyF and hlyR (Table 4.1) (final concentration 0.3 µM each) (Whitehead Scientific), 1U *Biotaq* DNA polymerase (Bioline), 1 µl crude extract ($10^0$). Amplification was carried out in a thermal cycler GeneAmp® PCR system 2700 (Applied Biosystems) with the following programme: Initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 40 s and a final extension step at 72°C for 5 min. The PCR products underwent electrophoresis on a 1.5% agarose D-1 LE gel (Whitehead Scientific) and visualized by staining with ethidium bromide. The amplified PCR products were viewed using the Alphaimager® HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC™ software version 4.0.0.



**Table 4.1** Sequence of the primer set specific for the amplification of the *hly* gene of *L. monocytogenes* (Blais and Phillippe 1995)

| Description | Primer sequence | Amplicon size |
|---|---|---|
| Primer set specific for the amplification of the *hly* gene of *L. monocytogenes* | **hlyF**: 5' CAT TAG TGG AAA GAT GGA ATG 3'<br>**hlyR**: 5' TCG ATC ACT CTG GAG GAT AC 3' | 730 bp |

### 4.3.3 Sequencing

The PCR products of the *hly* gene for all the referenced serotypes (including isolates from different geographic locations with the same serotypic designation) were sent for purification and sequencing at the Stellenbosch DNA sequencing unit at Stellenbosch University.

### 4.3.4 RFLP analysis



Subsequent to PCR and sequencing, it was observed from the sequencing results that individual digest profiles from restriction enzyme analysis could not be established for every serotype of *L. monocytogenes*. Instead of there being SNPs present to differentiate between each serotype, there were SNPs conserved and shared between groups of serotypes. As a result, *L. monocytogenes* serotypes could be placed into lineage groups by RFLP analysis. Where the SNP was conserved in a region of the gene and common among the serotypes of one lineage group and where it was part of an enzyme recognition site, then an enzyme that cleaved at that site was selected for RFLP analysis (Table 4.2). PCR products were digested by using the enzymes selected for each lineage group.

**Table 4.2** The enzymes used to perform RFLP analysis in the *hly* gene where a SNP was present and common to more than one serotype in a lineage group

| Gene | Serotype | Lineage group | Enzyme | Cut site | Band sizes |
|---|---|---|---|---|---|
| *hly* | 1/2b, 3b, 4b, 4d, 4e, 7 | I | *Nde*I Fastdigest (Fermentas) | **C**A/TATG | 410; 320 |
| | 1/2a, 1/2c, 3a, 3c | II | *Hae*II (Fermentas) | RGCGC/Y<br>**A**GCGCT (**cuts all serotypes**)<br>**G**GCGC/Y (**SNP**) | 455; 274<br>278; 274; 178 |
| | 4a, 4c | III | *Bsh*1285I Fastdigest (Fermentas) | **C**GRY/CG | 390; 340 |

nucleotides highlighted in bold indicate the SNP

r = purine

y = pyrimidine



### 4.3.5 MAMA-PCR

#### 4.3.5.1 Reference strains

The DNA was extracted as previously described from all the referenced *L. monocytogenes* serotype cultures in the culture collection, and used for MAMA-PCR.

#### 4.3.5.2 Primer sequences

Mismatch primers; lmaF, lmbR, lmcR and lmdF were designed according to Jinneman and Hill (2001), which include intentional mismatches to distinguish between *L. monocytogenes* lineage groups (Table 4.3). This protocol, however, was modified to include primer sequences hlyF and hlyR described by Blais and Phillippe (1995) which amplify a 730 bp region of the *hly* gene for all *L. monocytogenes* serotypes. For the mismatch primers, the SNP which distinguishes between lineage groups are included in the 3' end of the primer sequence. This renders *Taq* polymerase unable to extend the primer (Qiang *et al*. 2002). The mismatch primer is paired with hlyF or hlyR (Table 4.4) to produce a PCR product with a band size specific for a single lineage group.



#### 4.3.5.3 PCR amplification

PCR amplification was performed with the same PCR facilitators, concentrations and parameters as described earlier.

**Table 4.3** *L. monocytogenes* MAMA-PCR primer sequences (The primer sequences for lma, lmb, lmc and lmd were adapted from Jinneman and Hill 2001)

| **Primer** | **Primer sequence 5'- 3' (including intentional mismatches)[a]** | ***L. monocytogenes* lineage type specified** |
|---|---|---|
| hlyF | CAT TAG TGG AAA GAT GGA ATG | I, II, III |
| hlyR | TCG ATC ACT CTG GAG GAT AC | I, II, III |
| lmaF | AAG CCG TAA TTT ACG G*T*G **A**C | I |
| lmbR | GTA AGT CTC CGA GGT T*G*C **A**A | III |
| lmcR | GAA CTC CTG GTG TTT C*T*C **A**A | II |
| lmdF | CAC CAG GAG TTC CCA TTG **A**C | I |

[a] Bold nucleotides indicate intentional mismatches introduced into the sequence. Italicized and underlined nucleotides are unique to *L. monocytogenes* sequence type specified in column three. Italicized nucleotides indicate the nucleotide is unique to *L. monocytogenes* sequence type in column three and one other *L. monocytogenes* sequence type. Other nucleotides are common for all *L. monocytogenes* lineages.



**Table 4.4** *L. monocytogenes* MAMA-PCR primer combinations and predicted amplification results

| **Primer set** | **Primers** | **Amplicon size** | **Lineage I** | **Lineage II** | **Lineage III** |
|---|---|---|---|---|---|
| A | hlyF and hlyR | 730 bp | + | + | + |
| B | lmaF and hlyR | 269 bp | + | - | - |
| C | hlyF and lmbR | 529 bp | - | - | + |
| D | hlyF and lmcR | 580 bp | - | + | - |
| E | lmdF and hlyR | 161 bp | + | - | - |

## 4.4 RESULTS AND DISCUSSION

The sequencing data of the PCR products were analyzed by importing the nucleotide sequences into the BioEdit programme. Once the nucleotide sequences of the serotypes were aligned (for the *hly* gene) it was discovered that there were conserved regions shared among certain serotypes of *L. monocytogenes*. These conserved regions had SNPs that were shared among serotypes and on this basis the serotypes could be classified into lineage groups by RFLP analysis as opposed to obtaining single digest profiles for all serotypes which seemed unlikely due to the scarcity of SNPs and RFLPs in the *hly* gene (Annexure 2-4). The frequency with which SNPs occur over the genome is surely much greater than that of RFLPs alone (Schork *et al.* 2000). There is no published literature to date that shows the RFLP technique effective to discriminate between all the serotypes of *L. monocytogenes*, generating a digest profile for each serotype. Alternative sub-typing techniques including PCR where five primer sets were designed to classify strains into three serotype groups namely 1/2a(3a), 1/2b(3b) and 4b(d,e) (Borucki and Call 2003; Shen *et al*. 2006) indicates that discrimination between all serotypes even by alternative approaches have not been established. Once all the sequenced PCR products were analyzed in this study, it was possible to characterize the serotypes into 3 lineage/serotypic groups on the basis of the SNPs that are common to serotypes in the conserved region of the sequence. Serotypes 1/2b, 3b, 4b, 4d, 4e and 7 were characterized as lineage I isolates; serotypes 1/2a, 1/2c, 3a and 3c were characterized as lineage II isolates and serotypes 4a and 4c were characterized as lineage III isolates (Table 4.2).

While lineage III isolates initially embodied serotypes 4a and 4c, recent characterization of isolates in this lineage revealed that an atypical serotype 4b was also included in lineage III (Roberts *et al*. 2006; den Bakker *et al*. 2008; Ragon *et al*. 2008). This type of lineage classification complies with other research that placed the same serotypes into lineage groups on the basis of other genotypic testing (Rasmussen *et al*. 1995; Moorhead *et* al. 2003; Ragon *et al*. 2008).

All reference strains were subjected to PCR for the amplification of a 730 bp region of the *hly* gene specific for all *L. monocytogenes* isolates regardless of lineage group classification (Figure 4.1). Included as negative controls were other bacterial strains *Escherichia coli*, *Salmonella* serovar Enteritidis and 3 other species of the genus *Listeria*, namely *L. welshimeri*, *L. grayi* and *L. innocua*. There was no amplification of a 730 bp gene fragment in these organisms proving that the *hly* gene fragment is specific to *L. monocytogenes*. Following PCR, the DNA extracted from the reference cultures was subjected to RFLP analysis (Table 4.2 / Figure 4.2 and Figure 4.3) and the expected digest profile was obtained for lineage group classification of serotypes. All 12 serotypes were digested with enzyme *Nde*I, although, only *L. monocytogenes* serotypes 1/2b, 3b, 4b, 4d, 4e and 7 were digested to produce a 410 bp and 320 bp fragment / band size whereas the other serotypes remained uncut. These serotypes were characterized as lineage I isolates as grouped by other researchers by alternative genotyping methods. All 12 serotypes were digested with enzyme *Hae*II. Since the enzyme cuts where a purine (A or G) is present, two digest profiles were generated. Where nucleotide G (SNP) is

present, serotypes 1/2a, 1/2c, 3a and 3c are digested to produce three bands, namely; 278 bp, 274 bp and 178 bp. Since the 274 bp and 278 bp fragments are basically identical in size, they often appear as one band on the gel. It is important to note that enzyme *Hae*II cuts within all serotypes where nucleotide A is present, generating 2 band sizes of 455 bp and 274 bp. However where the SNP is present, the enzyme cuts within the 455 bp fragment generating 278 bp and 178 bp band sizes. Thus, the absence of the 455 bp fragments and the presence of the 178 bp fragments are indicative of a lineage II isolate. All 12 serotypes were cut with enzyme *Bsh*1285I and only 2 serotypes were digested with this enzyme, namely serotypes 4a and 4c generating two fragments of 390 bp and 340 bp. These isolates were classified as lineage III isolates. The other serotypes remained uncut.

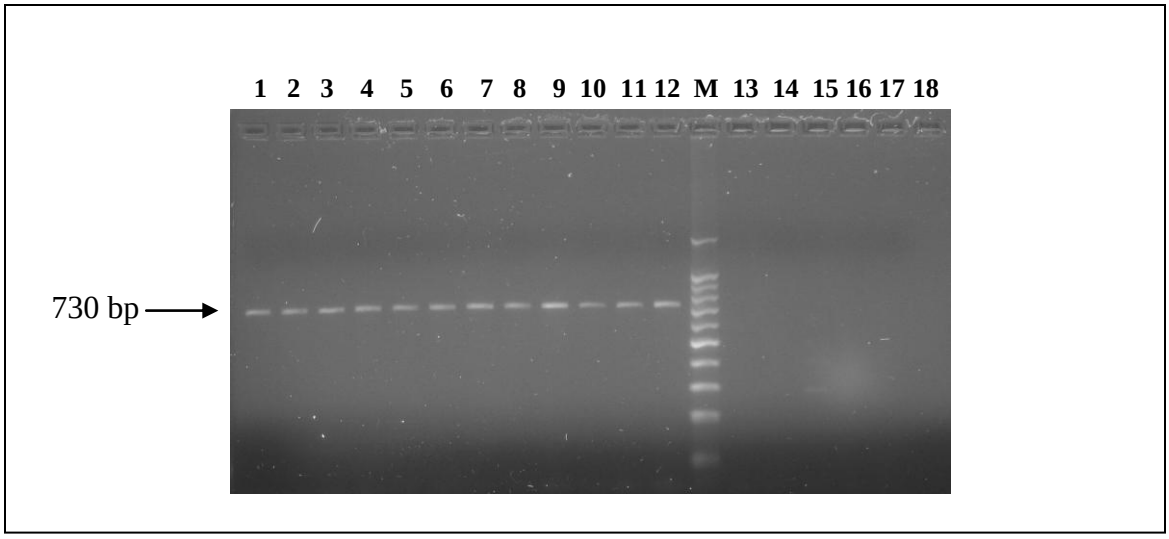


**Figure 4.1** PCR amplification products of the *hly* gene of the 12 serotypes of *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 7) and other bacterial species. **Lane 1**: *L. monocytogenes* 1/2a; **lane 2**: *L. monocytogenes* 1/2b; **lane 3**: *L. monocytogenes* 1/2c; **lane 4**: *L. monocytogenes* 3a; **lane 5**: *L. monocytogenes* 3b; **lane 6**: *L. monocytogenes* 3c; **lane 7**: *L. monocytogenes* 4a; **lane 8**: *L. monocytogenes* 4b; **lane 9**: *L. monocytogenes* 4c; **lane 10**: *L. monocytogenes* 4d; **lane 11**: *L. monocytogenes* 4e; **lane 12**: *L. monocytogenes* 7; **M**: 100 bp DNA ladder (Promega); **lane 13**: *L. welshimeri*; **lane 14**: *L. innocua*; **lane 15**: *L. grayi*; **lane 16**: *Escherichia coli*; **lane 17**: *Salmonella enteritidis*; **lane 18**: negative control (water).

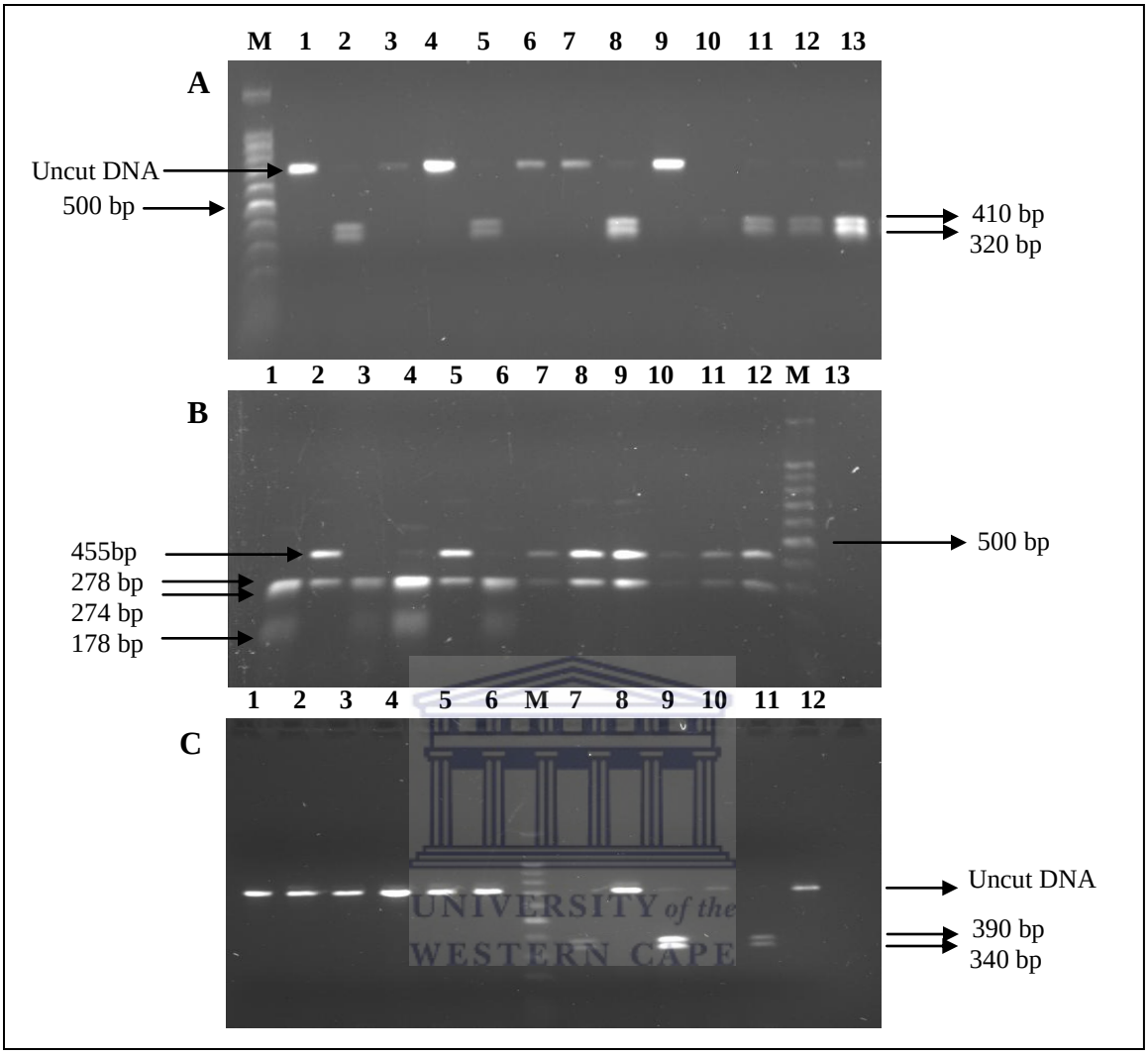


**Figure 4.2** RFLP analysis of the 12 serotypes of *L. monocytogenes* for lineage group identification with enzymes (**A**) *Nde*I, (**B**) *Hae*II and (**C**) *Bsh*1285I. **M**: 100 bp DNA ladder (Promega). **A1**: *L. monocytogenes* 1/2a; **A2**: *L. monocytogenes* 1/2b; **A3**: *L. monocytogenes* 1/2c; **A4**: *L. monocytogenes* 3a; **A5**: *L. monocytogenes* 3b; **A6**: *L. monocytogenes* 3c; **A7**: *L. monocytogenes* 4a; **A8**: *L. monocytogenes* 4b; **A9**: *L. monocytogenes* 4c; **A10**: *L. monocytogenes* 4d; **A11**: *L. monocytogenes* 4e; **A12**: *L. monocytogenes* 7; **A13**: *L. monocytogenes* 7 (repeated); (**B**) **B1**: *L. monocytogenes* 1/2a; **B2**: *L. monocytogenes* 1/2b; **B3**: *L. monocytogenes* 1/2c; **B4**: *L. monocytogenes* 3a; **B5**: *L. monocytogenes* 3b; **B6**: *L. monocytogenes* 3c; **B7**: *L. monocytogenes* 4a; **B8**: *L. monocytogenes* 4b; **B9**: *L. monocytogenes* 4c; **B10**: *L. monocytogenes* 4d; **B11**: *L. monocytogenes* 4e; **B12**: *L. monocytogenes* 7; **B13**: negative control (water); (**C**) **C1**: *L. monocytogenes* 1/2a; **C2**: *L. monocytogenes* 1/2b; **C3**: *L. monocytogenes* 1/2c; **C4**: *L. monocytogenes* 3a; **C5**: *L. monocytogenes* 3b; **C6**: *L. monocytogenes* 3c; **C7**: *L. monocytogenes* 4a; **C8**: *L. monocytogenes* 4b; **C9**: *L. monocytogenes* 4c; **C10**: *L. monocytogenes* 4d; **C11**: *L. monocytogenes* 4a (the DNA of *L. monocytogenes* 4e was erroneously not loaded onto the gel; however it was not cut with enzyme *Bsh*1285I – data not shown); **C12**: *L. monocytogenes* 7.

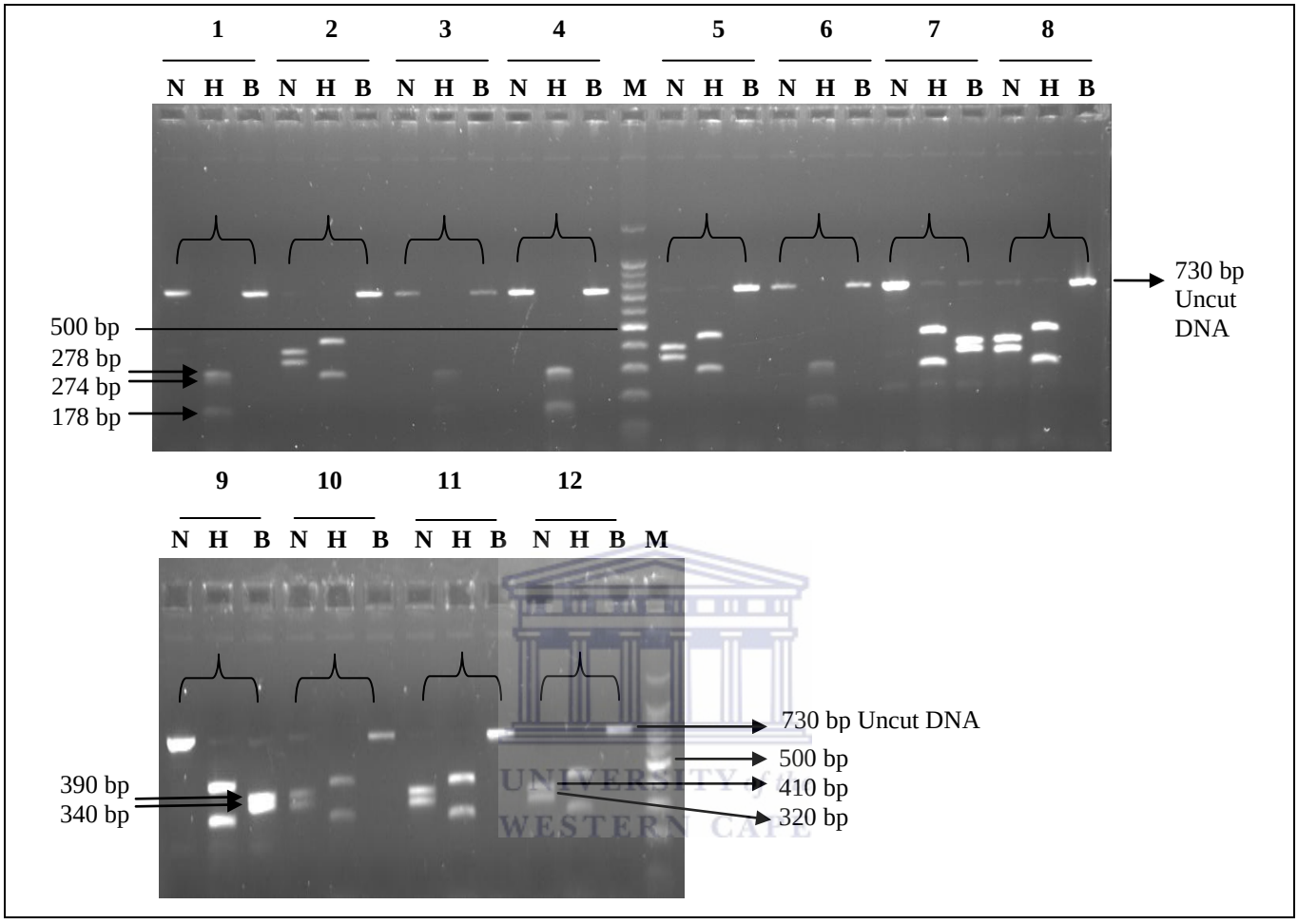


**Figure 4.3** RFLP analysis (another representation) of the 12 serotypes of *L. monocytogenes* for lineage group identification with enzymes **N** = *Nde*I, **H** = *Hae*II and **B** = *Bsh*1285I. Numbers 1-12 represent the 12 *L. monocytogenes* serotypes that are cut by the 3 enzymes. **M**: 100 bp DNA ladder (Promega); **lanes 1**: *L. monocytogenes* 1/2a; **lanes 2**: *L. monocytogenes* 1/2b; **lanes 3**: *L. monocytogenes* 1/2c; **lanes 4**: *L. monocytogenes* 3a; **lanes 5**: *L. monocytogenes* 3b; **lanes 6**: *L. monocytogenes* 3c; **lanes 7**: *L. monocytogenes* 4a; **lanes 8**: *L. monocytogenes* 4b; **lanes 9**: *L. monocytogenes* 4c; **lanes 10**: *L. monocytogenes* 4d; **lanes 11**: *L. monocytogenes* 4e; **lanes 12**: *L. monocytogenes* 7.

The MAMA-PCR assay was adapted from Jinneman and Hill (2001) and applied in this study to be able to distinguish between the 3 lineage groups of *L. monocytogenes*. The primers designed for the target sequence include a single mismatch but for the sequences of other lineages groups, a double mismatch. For MAMA-PCR all the *L. monocytogenes* reference strains were included in order to determine the accuracy of this technique (Figure 4.4).

Primer sequences hlyF and hlyR (Blais and Phillippe 1995) (primer set A) amplify a 730 bp region of the virulent *hly* gene inherent to *L. monocytogenes* regardless of lineage group and covers the area where SNPs are present for lineage group differentiation. Primers lmaF and hlyR (primer set B) amplify a 269 bp product specific for lineage I isolates. The SNP in the fourth position from the 3' end (designated in italics) of lmaF is specific for the lineage I isolates (Table 4.3) and one other lineage group, namely lineage III whereas the final nucleotide (SNP) of the primer sequence in the 3' position is specific only for lineage I. Primers hlyF and lmbR (primer set C) amplify a 529 bp product specific for lineage III isolates. The SNP in the fourth position from the 3' end (designated in italics) of lmbR is specific for the lineage III isolates and one other lineage group, namely lineage I whereas the final nucleotide (SNP) of the primer sequence in the 3' position is specific only for lineage III isolates. Primers hlyF and lmcR (primer set D) amplify a 580 bp product specific for lineage II isolates. The SNP in the fourth position from the 3' end (designated in italics) as well as the last nucleotide (SNP) of lmcR is specific for the lineage II isolates only. Primers lmdF and hlyR (primer set E) amplify a

161 bp product specific for lineage I isolates. The final nucleotide (SNP) of the primer sequence in the 3’ position is specific only for lineage I isolates.

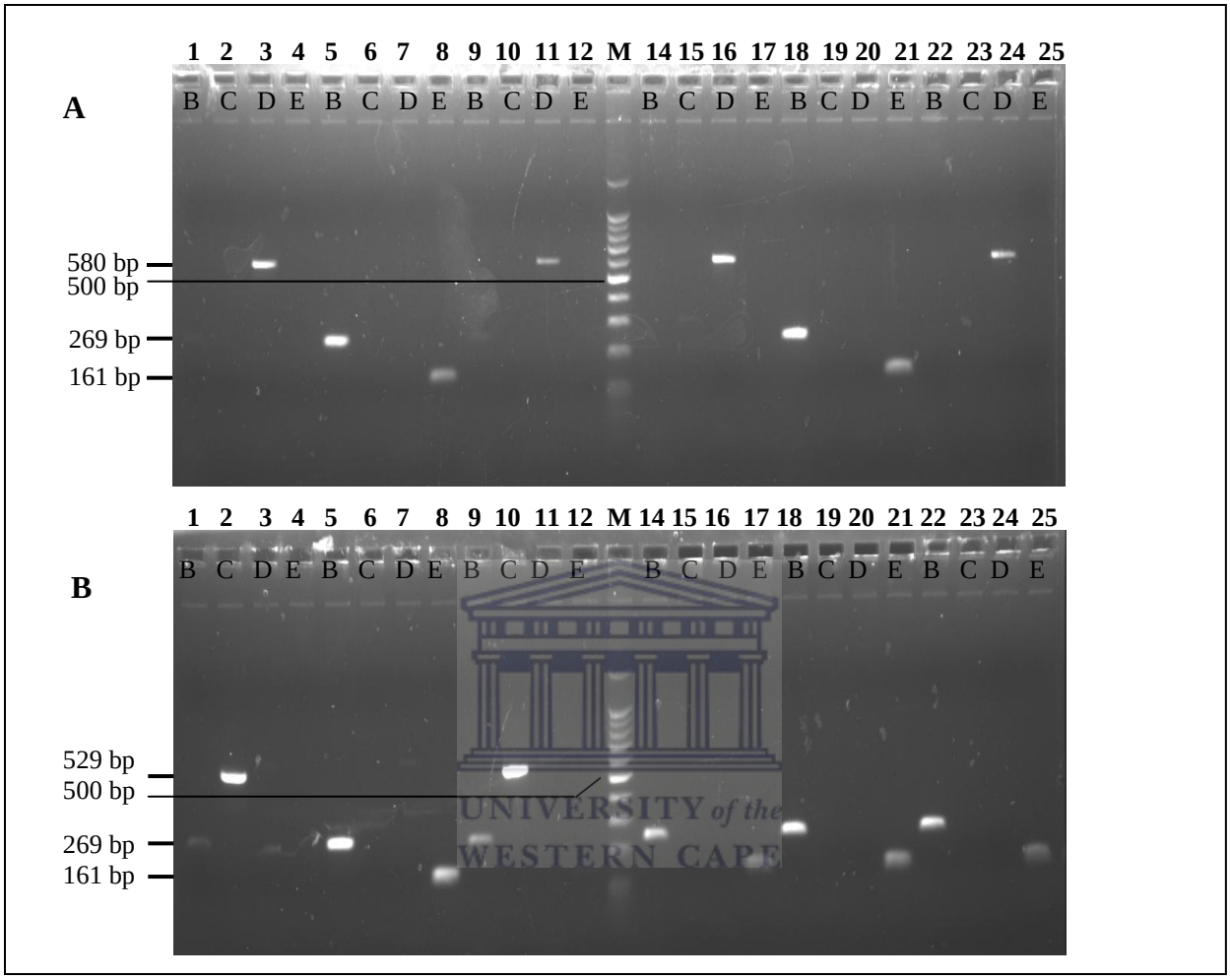




**Figure 4.4** MAMA-PCR amplification results for the *L. monocytogenes* reference strains where **B** represents primer set B: lmaF and hlyR, **C** represents primer set C: hlyF and lmbR, **D** represents primer set D: hlyF and lmcR and **E** represents primer set E: lmdF and hlyR. **M**: 100 bp DNA ladder (Promega); (**A**) **lanes 1-4**: *L. monocytogenes* serotype 1/2a; **lanes 5-8**: *L. monocytogenes* serotype 1/2b; **lanes 9-12**: *L. monocytogenes* serotype 1/2c; **lanes 14-17**: *L. monocytogenes* serotype 3a; **lanes 18-21**: *L. monocytogenes* serotype 3b; **lanes 22-25**: *L. monocytogenes* serotype 3c. (**B**) **Lanes 1-4**: *L. monocytogenes* serotype 4a; **lanes 5-8**: *L. monocytogenes* serotype 4b; **lanes 9-12**: *L. monocytogenes* serotype 4c; **lanes 14-17**: *L. monocytogenes* serotype 4d; **lanes 18-21**: *L. monocytogenes* serotype 4e; **lanes 22-25**: *L. monocytogenes* serotype 7.

*L. monocytogenes* serotypes 1/2a, 1/2c, 3a and 3c were amplified when only primer set D was used, producing an amplicon of 580 bp indicative of lineage II isolates. *L. monocytogenes* serotypes 1/2b, 3b, 4b, 4d, 4e and 7 were all amplified with primer sets B and E producing amplicons of 269 bp and 161 bp respectively, indicative of lineage I isolates. *L. monocytogenes* serotypes 4a and 4c were the only two isolates amplified with primer set C, producing a 529 bp amplicon, indicative of lineage III isolates. The characterization of *L. monocytogenes* isolates into lineage groups the MAMA-PCR technique confirms that the method of PCR-RFLP analysis designed and employed in this study is effective, works and can be applied as an alternative approach for lineage group classification of *L. monocytogenes* serotypes.



It is important to mention that the PCR-RFLP technique may be more effective and discriminatory for lineage group analysis since a banding profile will only result should the SNP restriction site be present. Thus, there will be no ambiguity when interpreting results. Secondly, the *hly* gene of *L. monocytogenes* undergoes RFLP analysis and as indicated earlier, this gene is inherent to *L. monocytogenes*, which is relevant since results will be conclusive for *L. monocytogenes*. On the other hand, where MAMA-PCR is concerned, it has been observed that the presence and size of the bold amplicon is the indicator for the lineage group characterization of isolates. The way the primer is designed with only the SNP and an intentional mismatch in the 3' end of the primer to differentiate between lineage groups may lead to the amplification of isolates of other lineage groups, even though this amplification is weaker and is considered a 'non-

specific.' For example, with reference to Figure 4.4, *L. monocytogenes* serotype 4c (lanes 9-12 B) was amplified with primer set C, since it is a lineage III isolate. However, a fainter amplicon was produced with primer set B, a 269 bp amplicon characteristic for a lineage I isolate but no amplification resulted with primer set E (also to distinguish a lineage I isolate). This 'non-specific' band could be a result of the primer set annealing to and amplifying the region used to distinguish lineage I isolates, even though the SNP for lineage I isolates was not present. It may be possible that *Taq* polymerase was able to extend the region past the SNP. This is a referenced 4c isolate so the expected result was that amplification would only occur with primer set C; however a limitation to using this technique may result especially when applying it to food isolates, where ambiguous results my lead to the incorrect interpretation thereof.



The principle of MAMA-PCR is that a single mismatch in the MAMA primer at the third nucleotide from the 3' end does not influence the yield of the PCR product whereas the incorporation of the intentional mismatch is to inhibit the reaction (Qiang *et al*. 2002).

## 4.5 CONCLUSION

The PCR-RFLP technique was developed and applied to the virulent *hly* gene of *L. monocytogenes* that contained suitable polymorphisms which allowed for distinction between lineage groups. PCR amplified gene products were cleaved with restriction enzymes *NdeI*, *Hae*II and *Bsh*1285I. These enzymes were selected since they had the suitable SNP restriction site. The fragments generated allowed distinction between *L. monocytogenes* isolates and placed isolates into lineage groups I, II or III. This method of lineage group distinction by RFLP analysis proved successful and no non-specific bands were obtained. PCR-RFLPs characterized *L. monocytogenes* serotypes 1/2b, 3b, 4b, 4d, 4e and 7 as lineage I isolates; *L. monocytogenes* serotypes 1/2a, 1/2c, 3a and 3c as lineage II isolates and *L. monocytogenes* serotypes 4a and 4c as lineage III isolates. Since the species of *Listeria* may co-exist in a food or environmental sample, especially *L. innocua* and *L. monocytogenes*, PCR amplification of the *hly* gene which is specific to *L. monocytogenes*, means the PCR-RFLP method does not have to be applied to any other *Listeria* spp. since ambiguous results will not occur. *L. monocytogenes* is the only species of the genus that causes disease in humans and the interpretation of PCR-RFLP results will be specific for *L. monocytogenes* only. This is important since this method will be applied to *L. monocytogenes* DNA isolated from food, environmental, animal and clinical samples.

The distinction between serovars is necessary and important in order to have a better understanding of the virulence factors, the levels of expression of virulence factors and epidemiology of human listeriosis. The MAMA-PCR approach was selected as an additional sub-typing approach in order to determine whether the sub-typing discrimination obtained by PCR-RFLP was conclusive. The use of two or more sub-typing procedures are often more powerful and discriminatory than the application of the single approach. The results obtained with the MAMA-PCR technique was in agreement with the results obtained with PCR-RFLP analysis allowing the PCR-RFLP technique to be applied to naturally contaminated *L. monocytogenes* samples.

# CHAPTER 5

# RFLP analysis for lineage group classification of *Listeria monocytogenes* serotypes isolated from food samples, chicken blood and human clinical isolates

## 5.1 ABSTRACT

**Aim:** Food and environmental *Listeria monocytogenes* isolates as well as isolates from human cases of listeriosis, were analyzed using RFLP analysis for lineage group identification. This information would determine the group of serotypes adapted to the food processing environment and those involved in clinical cases.

**Materials and Methods:** *L. monocytogenes* isolates enumerated from food, environmental and human listeriosis cases were subjected to PCR-RFLP analysis and MAMA-PCR. These molecular techniques characterized the DNA isolates as either lineage type I, II or III. For RFLP analysis, enzymes; *Nde*I, *Hae*II and *Bsh*1285I were selected for lineage group distinction based on SNPs that were shared and conserved among groups of serotypes. This method of lineage group distinction, characterized all isolates from seven clinical samples as lineage I, whereas, from a total of 165 isolates collected from the food and the food-processing environment, lineage I and II accounted

for 21% and 79% respectively. There was no representation of lineage III isolates from samples analyzed in this study.

**Conclusion:** Based on *hly* PCR-RFLP analysis, lineage group characterization of *L. monocytogenes* isolates could be performed from all samples acquired for this study.

**Significance and impact of study:** The PCR-RFLP sub-typing technique has provided a reliable and reproducible method to screen and categorize *L. monocytogenes* isolates by lineage type based on conserved nucleotide differences in the *hly* gene. This technique was applied to *L. monocytogenes* isolates enumerated from food, environmental and human clinical samples and may be vital in determining the pathogenic potential of isolates.

## 5.2 INTRODUCTION

*Listeria monocytogenes* is a ubiquitous, gram-positive, facultative, intracellular food-borne pathogen that is responsible for an invasive illness, namely listeriosis. Although the incidence of infection is rare, the hospitalization and mortality rates are rather high (≥ 90% and 20-30% respectively) (Jemmi and Stephan 2006; Chen and Knabel 2008; Esteban *et al*. 2009; Ward *et al*. 2010). These statistics make listeriosis infection a public health concern. Food-borne listeriosis generally has a long incubation time varying from a few days (seven) to two to three months which makes source tracking difficult and outbreaks are easily missed (Yde and Genicot 2004; Chen *et al*. 2005). Listeriosis cases mainly result from the consumption of contaminated foods; with RTE foods that permit the growth of *L. monocytogenes* appearing to be responsible for most listeriosis cases (Carminati *et al.* 2004; Rip and Gouws 2009; Ward *et al*. 2010). The strain of *L. monocytogenes* as well as the host's immune systems defense determines their response to disease therefore an infective dose for *L. monocytogenes* cannot be clearly defined and as a result all contaminated RTE food poses a risk for contamination and illness and is seen as a hazard (Vasilev *et al*. 2010). The risk of infection does however, increase in relation to dose size and there were incidences of infection related to fewer than 100 cfu $g^{-1}$ of food (Willis *et al*. 2006). The types of foods as vehicles of infection have increased due to changes in food production, preservation and consumption (Yde and Genicot, 2004).

*L. monocytogenes* strains are able to persist in food processing environments and during storage of foods due to its ability to grow at refrigeration temperature (it displays psychrotrophic and microaerophilic properties), in high salt concentrations and foods with a low pH. As a result of the ability of *L. monocytogenes* to survive and grow under conditions that are considered unfavourable for other food-borne microorganisms, the contamination of foods by this organism has been the leading cause of food recalls (Ducey *et al*. 2007; Ward *et al*. 2010). *L. monocytogenes* biofilm formation makes this species highly resistant to cleaning and disinfection procedures. For this reason, this organism tends to persist in the food processing environment and the complete eradication of this organism is almost impossible (Dabrowski *et al*. 2003; Dharmarha 2008).



In the elderly and those with an impaired immune system, listeriosis may cause meningitis, septicaemia and encephalitis whereas in healthy individuals, food-borne transmission of *L. monocytogenes* may cause acute febrile gastroenteritis (Tran and Kathariou 2002; Roberts *et al*. 2006). Pregnant women also represent a high risk-group for *Listeria monocytogenes* infection (Abram *et al*. 2003). *L. monocytogenes* targets and crosses the placental barrier in pregnant women. The crossing of the placental barrier leads to serious fetal infections, fetal death, miscarriages, premature births and neonatal infections (Longhi *et al*. 2003; Todar 2008; Schett *et al*. 2005). Listeriosis cases may be under-reported in many countries due to the absence of adequate surveillance programmes (Garrido *et al*. 2008).

For the average healthy person, the risk of becoming ill with listeriosis is quite minimal since scientific evidence has indicated that *Listeria* are generally consumed with no ill effects. As a result, the European Union (EU) legislation is in agreement with a limit of 100 cfu $g^{-1}$ *L. monocytogenes* in certain food products at the end of shelf life of RTE foods whereas the United States is more stringent and has favoured a zero tolerance ruling. It has been suggested that the best way to control *L. monocytogenes* is through monitored preventative measures and appropriate controls (Borucki *et al*. 2004; Curtis 2007; CFA 2010).



Isolates from human listeriosis cases in the Western Cape were included in this study. The identification and investigation of isolates involved in human cases of listeriosis require knowledge of the microbiological characterization of clinical and environmental isolates (Yde and Genicot, 2004). There are various concepts that must be considered when examining epidemiological data. According to Chen *et al*. (2005), "an outbreak is defined as an acute appearance of a cluster of an illness caused by a source strain that occurs in numbers in excess of what is expected for that time and place". Outbreaks can also be caused by multiple strains (van Belkum *et al*. 2007). Where that same source strain persists for a long time and is the cause of numerous outbreaks, an epidemic may occur.

Compared to countries that have developed surveillance systems for listeriosis, it has been reported that there are few or even no reports from Africa, Asia and South America

(Rocourt and Cossart 1997; Lemes-Marques *et al*. 2007). No specific national surveillance for listeriosis is conducted in South Africa (NICD 2007). According to the NICD report (2007) which provides up-to-date information on communicable diseases in South Africa, 5 culture confirmed cases of human listeriosis by *L. monocytogenes* were identified in the Eastern Cape region between December and January 2007. Symptoms of meningitis were displayed and all patients died within one to three days of hospitalization. The age of the patients ranged from 26 days to 46 years, two of which were HIV-positive and the others had compromised immune systems. In the KwaZulu Natal region, four cases were reported between January and February 2007 of which the clinical outcomes were not confirmed. Cases included a three month old infant, two adults in their twenties with a compromised immune system and an elderly diabetic. A shortage of resources for adequate investigations of disease outbreaks leads to the difficulty in controlling *L. monocytogenes* illness. Since there is an absence of data or surveillance of listeriosis in Africa, part of this research aims to 'fill the gap' and report on the *L. monocytogenes* serotypes involved in listeriosis in the Western Cape region. This study will form the basis for generating novel information about the distribution and ecology of *L. monocytogenes* strains and lineage groups in food and their association with clinical cases and also pave the way for more research to be undertaken in the future, especially in Africa.



*L. monocytogenes* is frequently present in certain food products and outbreaks of listeriosis have been linked to food types such as coleslaw, vegetables, cabbage, soft

cheeses, milk, seafood products including salmon, cold-smoked fish and trout, meat products and poultry (Vaz-Velho *et al*. 2001; Tran and Kathariou 2002; Dabrowski *et al*. 2003; Mahmood *et al*. 2003; Carminati *et al*. 2004; Chou and Wang 2006; Jemmi and Stephan 2006; López *et al*. 2008; Ford 2010). *L. monocytogenes* can grow and multiply at refrigeration temperatures which is a concern when products with an extended shelf-life are contaminated, for example pâté and vacuum-packed meats. The numbers of *L. monocytogenes* may be few at the start of production and reach high numbers by the end of the shelf-life (Willis *et al*. 2006). Pregnant women have been advised to abstain from foods such as camembert, brie, chèvre, blue cheeses and pâté (Dawson *et al*. 2006). In this study, food and animal samples which tested positive for *L. monocytogenes* included guacamole, meat, rooibos tea leaves, milk, vegetables, seafood and chicken blood.



Guacamole is a food product prepared with raw fruits and vegetables including avocado, minced tomato and onions to which chilli, cilantro, lemon juice and table salt could be added (Arvizu-Medrano *et al*. 2001; Science Daily 2010). This food-type may be contaminated by food-borne pathogens such as *L. monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* by conditions that introduce the pathogens to food, including the manipulation of the food-type by the food handlers in its preparation. Avocado pulp is the main component of guacamole with the pH close to neutral and the high concentration of lipids. The risk associated with this food-type is that it is not heat treated prior to consumption (Arvizu-Medrano *et al*. 2001). Guacamole dip ingredients have been implicated in restaurant outbreaks in the United States where it accounts for one out of 25

food-borne disease outbreaks in restaurants for the last ten years. The fact that it is prepared in large batches results in a small amount of contamination leading to human illness (CBC News 2010; Walton 2010). For axample, avocado pulp distributed by San Diego companies to Mexican restaurants has also been linked to *L. monocytogenes* contamination and was recalled by the FDA (TechNews 2001).

Epidemiological analysis and population genetic studies of *L. monocytogenes* using sub-typing methods have been really vital in improving the understanding of how *L. monocytogenes* is transmitted from animals or the environment through foods to humans. As mentioned before, most literature report that serotype 4b is responsible for the majority of human cases of listeriosis and that of the 13 serotypes, do 1/2a, 1/2b and 1/2c predominate in the food processing environment. Lineage I isolates of *L. monocytogenes* are responsible for most sporadic cases of listeriosis as well as the majority of epidemic outbreaks and is therefore a public health concern. Since no data is available in Africa, the findings of this study will determine whether the serotype distribution in this region corresponds with that which is reported elsewhere and would indicate whether certain serotypes contribute to the real burden of disease in the human population.



The aim of this research was to (i) determine the distribution and ecology of the strains of *L. monocytogenes* in the food supply in and around the Western Cape by (ii) developing and implementing DNA sub-typing methods and (iii) generating serotype distribution data for all naturally contaminated food samples and food processing facilities tested.

Clinical strains were acquired from Groote Schuur Hospital in the Western Cape region to determine the *L. monocytogenes* serotypes involved in the human clinical cases of listeriosis and to determine whether there is a correlation between the strains adapted to the food and food processing environments and those involved in human clinical cases of listeriosis.

## 5.3 MATERIALS AND METHODS

### 5.3.1 Acquisition and sample preparation of naturally contaminated food and clinical samples

#### 5.3.1.1 Clinical isolates

In order to determine the incidence of listeriosis, hospitals and pathology labs were asked to report cases of human listeriosis for molecular characterization of the isolates. However, notification of clinical cases for listeriosis was only received from one Hospital (Groote Schuur) who kindly provided all the isolates of *L. monocytogenes* responsible for seven human listeriosis cases in the period October 2008-2010. Isolates were received on horse blood agar.



#### 5.3.1.2 Guacamole, water and environmental samples

*Listeria monocytogenes* positive guacamole samples were acquired from an avocado/gucamole processing plant in the Limpopo province that produces and supplies guacamole to retail outlets (Table 5.1). A total of 112 samples, packaged and ready to be transported to retail outlets were analyzed in this study. All samples came from different batches and were acquired mainly over a two year period during the years 2008 and 2009. Three water samples (effluent rinse water) sourced at drains outside the guacamole food

processing plant, were included and tested in this study. For *L. monocytogenes* enumeration, isolates were cultured on chromogenic media; namely RAPID *L. monocytogenes* (Bio-Rad laboratories) where blue colonies were indicative of a positive result for *L. monocytogenes*. The presumptive positive colonies for *L. monocytogenes* were resuspended in 10 ml TSB and incubated overnight at 37°C. The overnight cultures were subjected to a DNA isolation procedure followed by PCR.

#### 5.3.1.3 Vegetable, seafood samples and environmental samples



Food and environmental samples were acquired from a food manufacturing plant that supplied pre-packed RTE vegetable products and fresh salads in the Western Cape area to food retail outlets for their deli section or self-service salad bars (Table 5.1). The environmental samples tested in this study included the kitchen drains through which effluent rinse water passed, the spatula used for mixing the coleslaw and the trough used to mix the coleslaw. The *L. monocytogenes* positive seafood samples (n = 3) were provided from a seafood plant right next door to the food manufacturing plant for RTE salads and vegetables. These samples were cultured on chromogenic Listeria Precis media (Oxoid) and green-blue colonies surrounded by a halo were indicative of a positive result for *L. monocytogenes*. All samples represented different batches on different days and were acquired during the year 2009. The colonies positive for *L. monocytogenes* were resuspended in 10 ml TSB and incubated overnight at 37°C. The overnight cultures were subjected to a DNA isolation procedure followed by PCR.

#### 5.3.1.4 Milk sample

A milk sample that was subjected to a process of ultraviolet (UV) treatment (1000 J $L^{-1}$) and high temperature short time (HTST) pasteurization was acquired for testing and analysis. The milk sample (25 ml) was aseptically transferred to a sterile stomacher bag (Grade packaging Ltd) and pre-enriched in 225 ml Listeria enrichment broth (LEB) (Oxoid), supplemented with Listeria selective supplement UVM1 (Oxford formulation) (Oxoid) and incubated overnight at 37°C. Thereafter 0.1 ml was extracted, inoculated into 10 ml ½ Fraser broth (FB) (Oxoid) and shaken overnight (114 rpm) at 37°C. The overnight culture was then subjected to the DNA isolation procedure and PCR.



#### 5.3.1.5 Rooibos tea and Food 25

Rooibos tea leaves was tested at various stages in its production and the sample that tested positive for *L. monocytogenes* represented the final product that underwent the steam sterilization process, after which the tea is ready to be packaged and sent to retail outlets. The sample was collected in a sterile bag straight off the production line following the steam sterilization process. The tea (25 g) was aseptically removed and resuspended in 225 ml LEB and incubated overnight at 35°C after which 0.1 ml was extracted and inoculated into 10 ml ½ FB for overnight incubation at 37°C. A loopful of culture was streaked onto chromogenic RAPID L. mono media (Bio-Rad laboratories) and incubated at 37°C overnight. Food 25 was a food sample aquired from an outside

laboratory that does microbiological examinations of food for various industries and food companies. The origin of the food sample was unknown. The isolate was received on PALCAM selective agar (Oxoid) and various colones were selected and streaked onto chromogenic RAPID L. mono media (Bio-Rad laboratories) and incubated at 37°C overnight. Presumptive positive colonies of the Food 25 and the rooibos tea leaves samples were inoculated into TSB and after incubation at 37°C overnight, DNA extraction and PCR were employed.

**5.3.1.6 Meat samples**



Cultures sustained as glycerol stocks of *L. monocytogenes* isolated from meat products were made available for this study. All samples (n = 17) were obtained from the University of Stellenbosch which stored the isolates in glycerol stocks at -20°C. Cultures (100 μl) was resuspended in TSB (Oxoid) and grown overnight at 37°C (Norton and Batt 1999; Smith *et al*. 2001). Following the overnight growth, DNA was extracted from the cultures and subsequently amplified by PCR. Chicken blood samples were acquired from a chicken abattoir company in the Western Cape region of South Africa and received in sterile heparin (EDTA or lithium chloride) blood collection tubes. One viable and culturable *L. monocytogenes* isolate was enumerated by conventional plating and molecular techniques (a detailed description of this process is explained in depth in chapter 6).

### 5.3.2 DNA isolation from naturally contaminated samples

A method modified by Agersborg *et al*. (1997) was used for DNA isolation. Following the overnight enrichment, a 2.0 ml aliquot culture was transferred to an eppendorf tube. Cultures were centrifuged at 9000 x g for 10 min. The pellet was resuspended in 400 µl sterile distilled water to which 400 µl 2 % Triton-X-100 (BDH Chemicals Ltd) was added and the contents mixed for 5 s. This suspension was left at room temperature for 10 min, thereafter incubated at 100°C for 10 min and centrifuged at 9000 x g for 4 min. The supernatant was transferred to a sterile eppendorf tube and 1 µl of this crude cell lysate was used for PCR amplification.



### 5.3.3 PCR amplification

PCR amplification was specific for a 730 bp product of the *hly* virulent gene of *L. monocytogenes* (Blaise and Phillippe 1995). Careful optimization of constituent quantities as well as thermal cycling parameters took place. For a 25 µl reaction, the mixture contained: 1 X PCR buffer (final concentration) (Bioline), $MgCl_2$ (final concentration 5mM) (Bioline), dNTP's (final concentration 200 µM) (Roche Diagnostics), primers hlyF and hlyR (final concentration 0.3 µM each) (Whitehead Scientific), 1U *Biotaq* DNA polymerase (Bioline), 1 µl template DNA ($10^0$).

Amplification was carried out in a thermal cycler GeneAmp® PCR system 2700 (Applied Biosystems) with the following programme: Initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 40 s and a final extension step at 72°C for 5 min. The PCR products underwent electrophoresis on a 1.5% agarose D-1 LE gel (Whitehead Scientific) and visualized by staining with ethidium bromide. The amplified PCR products were viewed using the Alphaimager® HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC™ software version 4.0.0.


**5.3.4 RFLP analysis**

RFLP analysis was performed on the DNA isolated from various ecosystems (food products, the environment, humans and animals) with the enzymes for lineage group classification I, II and III. For a 10 µl restriction digest, the mixture contained; 5 µl PCR product, 1 X restriction buffer (final concentration) (Fermentas) and restriction enzyme (final concentration 1U/µl) (Fermentas). The DNA was digested with *Nde*I (FastDigest) at 37°C for 25 min, *Hae*II at 37°C for 16 h and *Bsh*1285I (FastDigest) at 37°C for 25 min. The reactions were inactivated at temperatures described by the manufacturer. The restriction digest products underwent electrophoresis on a 1.5% agarose D-1 LE gel (Whitehead Scientific) and visualized by staining with ethidium bromide. An uncut DNA sample was used as a negative control. The digested DNA was viewed using the

Alphaimager® HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC™ software version 4.0.0.

### 5.3.5 Mismatch amplification mutation assay (MAMA)-PCR

Mismatch primers; lmaF, lmbR, lmcR and lmdF (refer to chapter 4) which include intentional mismatches to distinguish between *L. monocytogenes* lineage groups were used to confirm the method of lineage group classification by RFLPs designed and employed in this study (Table 4.3). The MAMA-PCR technique was applied to DNA extracted from naturally contaminated food samples as well as human clinical samples.

## 5.4 RESULTS AND DISCUSSION

All clinical human *L. monocytogenes* isolates were received on TBA media supplemented with horse blood. Isolates displayed haemoltyic activity and were resuspended in TSB and grown overnight at 37°C in order to proceed with molecular characterization. Compared to selective agars like PALCAM and Oxford which are unable to differentiate between *Listeria* spp., chromogenic RAPID L. mono media (Bio-Rad laboratories) and Listeria Precise media (Oxoid) have been useful in differentiating the pathogenic *L. monocytogenes* from the other non-pathogenic species. This chromogenic media may possibly lead to the detection of *Listeria* in food samples three days sooner than traditional methods (Willis *et al*. 2006). Presumptive *L. monocytogenes* isolates from food that appeared as green-blue colonies with a halo from *Listeria* Precise media and blue colonies on RAPID L. mono media were selected for further molecular characterization. These presumptive positive *L. monocytogenes* isolates were grown up in TSB overnight at 37°C.



The *hly* gene is inherent to *L. monocytogenes* strains. The DNA extracted from all *L. monocytogenes* cultures incubated overnight was amplified by PCR to produce a 730 bp fragment of the *hly* gene (Blais and Phillippe, 1995). A total of 172 *L. monocytogenes* isolates had been enumerated from human clinical cases (n = 7), guacamole (n = 112), salad and vegetables (n = 13), seafood (n = 3), environmental (n = 16), meat (n = 17), rooibos tea (n = 1), milk (n = 1), chicken blood (n = 1) and Food 25 (n = 1, unknown

food sample from an outside laboratory). All samples tested positive for the *hly* gene by PCR amplification (Figure 5.1 and 5.2a-d).

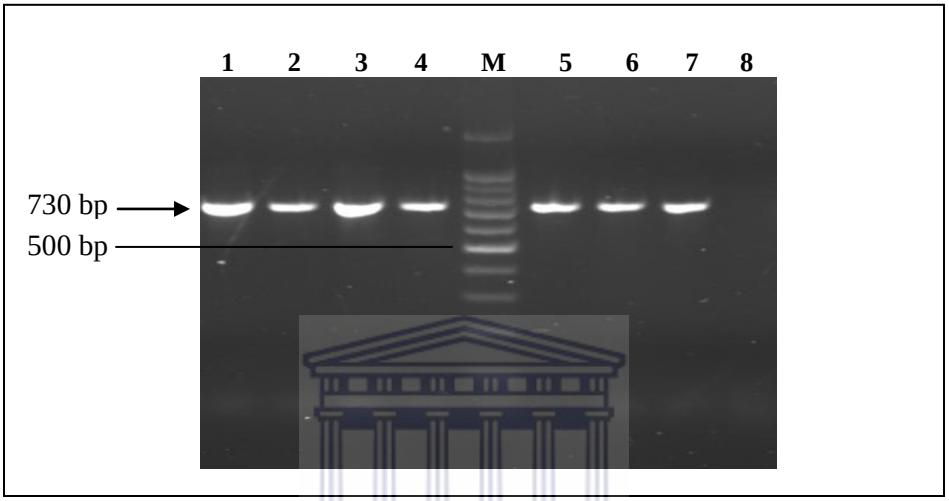




**Figure 5.1** PCR amplification of the *hly* gene of *L. monocytogenes* from isolates obtained from human clinical cases. **Lane 1**: clinical isolate 1; **lane 2**: clinical isolate 2; **lane 3**: clinical isolate 3; **lane 4**: clinical isolate 4; **M**: 100 bp DNA ladder (Promega); **lane 5**: clinical isolate 5; **lane 6**: clinical isolate 6; **lane 7**: clinical isolate 7; **lane 8**: negative control (water).

The acquisition of the guacamole samples from Limpopo has expanded the geographical representation among the isolates in this study. A total of 112 guacamole samples from different batches over a two year period were tested for *L. monocytogenes* (Figure 5.2a and Figure 5.2b). Avocado, a fruit, is a key ingredient of guacamole. This fruit can harbour *L. monocytogenes*, *Salmonella* spp. and *Esherichia coli* 0157:H7 depending on how they were harvested or processed (Gregerson 2009). Cross-contamination may result from one avocado to the next, through food-handlers or contaminated processing

equipment. Since *L. monocytogenes* is ubiquitous in nature and found in soil and other parts of the environment, *L. monocytogenes* that may be located on the skin of the avocado can be shifted to the flesh or interior of the fruit when it is cut (Gregerson 2009). In a study undertaken by Arvizu-Medrano *et al*. (2001), it was shown that the pH value of most avocado pulp was above 5.5 and the $a_w$ around 0.989 which are values suitable for the growth of most food-borne pathogens. Preventative measures, suggested by the authors, to ensure a level of safety for guacamole consumption include; washing of ingredients, contamination by food-handlers should be avoided during preparation and storage of the product, leftovers should be discarded and this food-product should be kept for no more than 3 h at room temperature or refrigeration temperature. Homemade guacamole, since not pasteurized, should be eaten within one to three days of preparation and refrigeration whereas store bought guacamole, though pasteurized should be eaten within five to seven days of opening. Although pasteurized, contamination may occur prior to packaging if proper sanitation protocols are not adhered to. Processing equipment, storage freezers and dripping water may contribute to the contamination of this food product post pasteurization and prior to packaging (Gregerson 2009).



According to research conducted by the CDC in the USA, it was discovered that in the restaurant deli industry, 4% of food related to disease outbreaks in the period 1998-2008 was linked to the consumption of contaminated fresh salsa or guacamole. These figures were apparently more than double that reported in the previous decade (Food Safety Watch 2010; Walton 2010). Avocados and guacamole should be seen as a vehicle for

food-borne infections caused by pathogens, including *L. monocytogenes*, as is seen in this study.

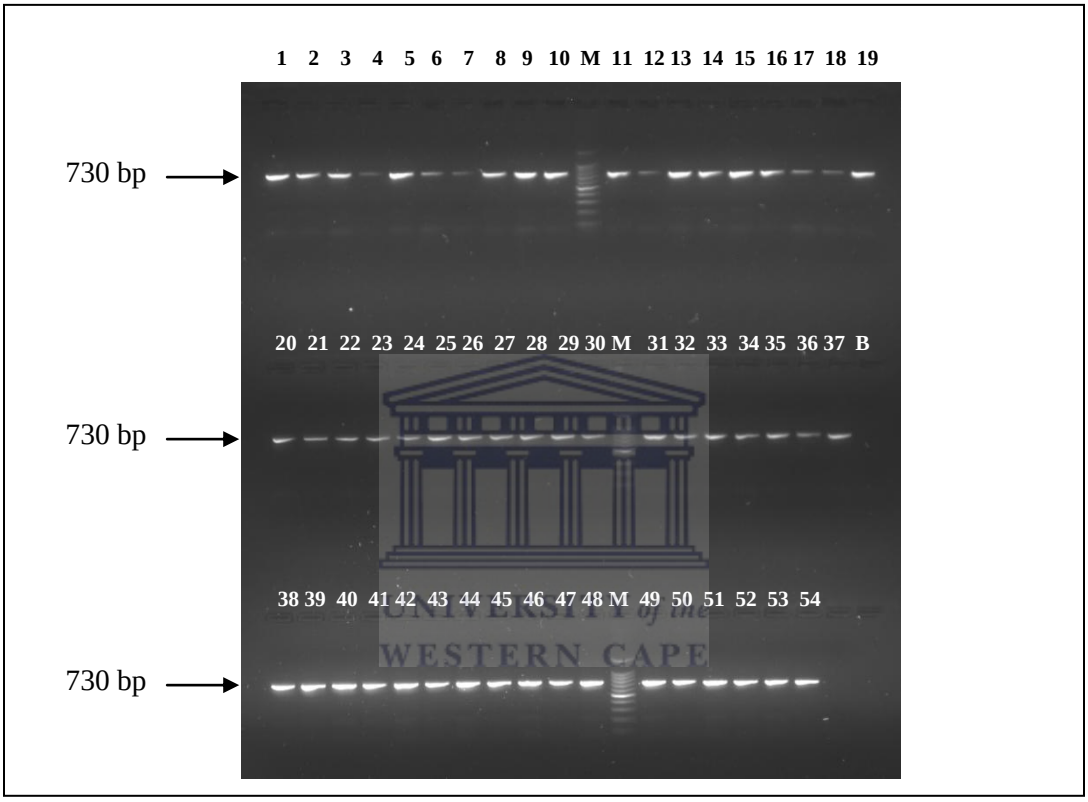


**Figure 5.2a** PCR amplification of the *hly* gene of *L. monocytogenes* from DNA isolated from guacamole samples. **M**: 100 bp DNA marker (Promega); **B**: negative control (water); **lanes 1-54**: *L. monocytogenes* DNA isolated and amplified from guacamole samples.

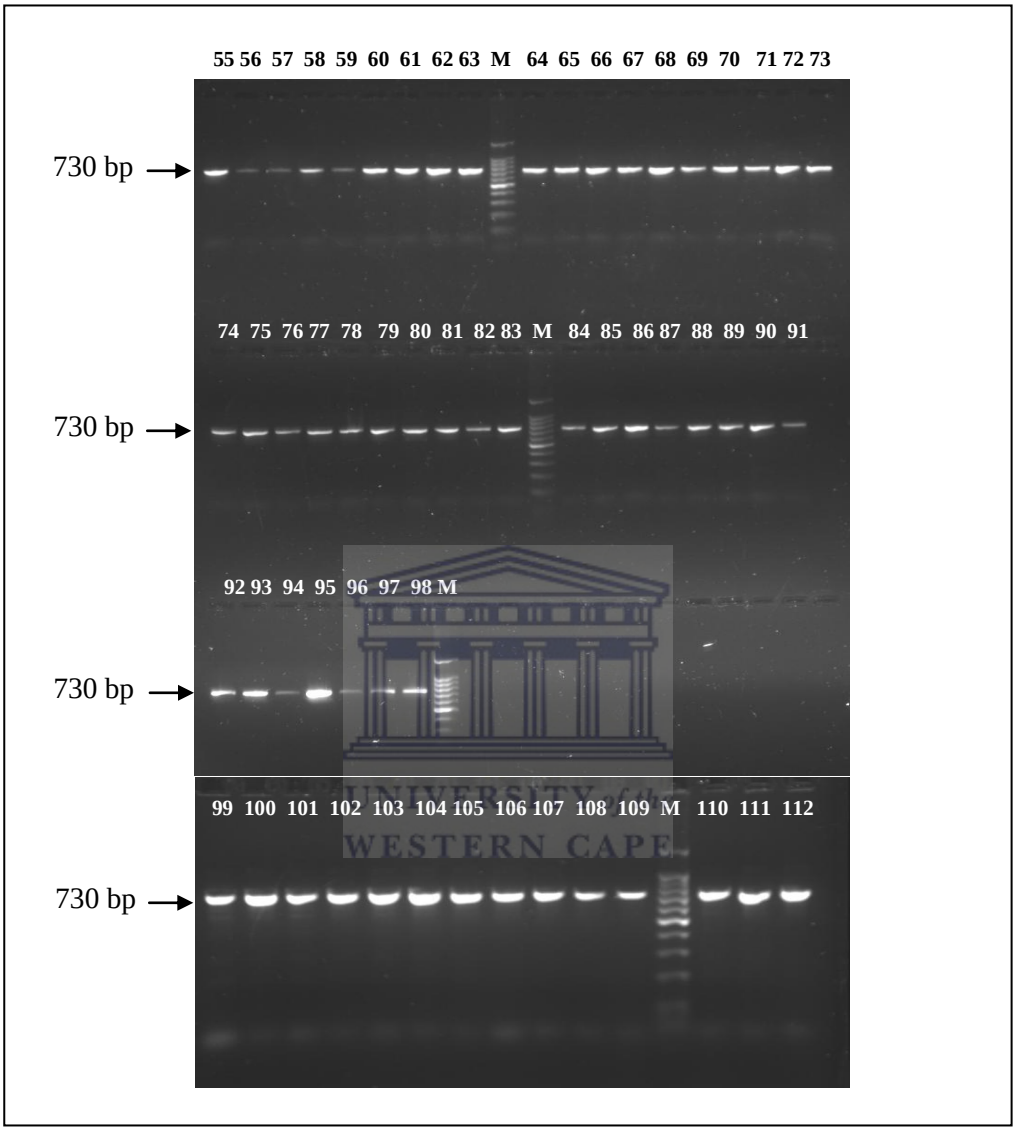


**Figure 5.2b** PCR amplification of the *hly* gene of *L. monocytogenes* from DNA isolated from guacamole samples. **M**: 100 bp DNA ladder (Promega); **B**: negative control (water); **lanes 55-112**: *L. monocytogenes* DNA isolated and amplified from guacamole isolates.

*L. monocytogenes* isolates from meat samples (n = 18) were obtained for this study from an outside source. Of these samples, 17 samples were positive for *L. monocytogenes*. Since the isolates were sustained as glycerol stocks over a period of time, there was no recollection of the type of meat sample from where it was isolated. DNA was extracted from all 17 cultures for PCR amplification of the *hly* gene (Figure 5.2c).

The salad and vegetable food samples (n = 13), which included carrots, coleslaw, Danish feta salad, spring onion, spelt and legume salad, hulled barley salad, Greek wheatberry salad (made with chickpeas), legume salad and barley salad were confirmed to be *L. monocytogenes* positive by traditional culture based methods and PCR amplification of the *hly* gene (Figure 5.2c). The list of food samples are mentioned in Table 5.1. A sample that tested positive for *L. monocytogenes* included spring onion, even after dipping in peroxyacetic acid of 150 ppm. Peroxyacetic acid is an ideal antimicrobial agent broadly effective against microorganisms and it breaks down in food to safe and environmentally friendly residues (acetic acid and hydrogen peroxide). As a result it is used in non-rinse applications. It is registered for use in dairy/cheese processing plants, on food processing equipment and in breweries and winery plants. It is also used as a disinfectant to control biofilm formation of bacteria (NOSB TAP materials database 2000). Contamination of the spring onion after rinsing with peroxyacetic acid could possibly be ascribed to cross contamination post this antimicrobial treatment. Processing equipment used to prepare the RTE salads and vegetables for retail outlets could be contaminated with *L. monocytogenes*. For example, such contamination has been been linked to coleslaw

(Farber and Peterkin 1991; Cocolin *et al*. 1997). Indeed, in this study, *L. monocytogenes* was isolated from environmental samples related to the processing facilities of the RTE food products tested. The environmental samples that tested positive for *L. monocytogenes* were representatives from the food processing facility that manufactured the guacamole and RTE salad and vegetables (Table 5.1). Environmental swabbing was performed in the food processing facility and samples were collected from the food processing equipment including; soup fillers (machine used to fill bags with soup), cabbage shredding machines, spatula for scooping coleslaw, a mixed trough which was used to mix coleslaw in and swabbing from the kitchen drains. From the guacamole processing facility, samples included effluent rinse water samples from three drains outside the plant, where effluent rinse water accumulates (Figure 5.2c and Figure 5.2d).



Where *L. monocytogenes* was detected in the kitchen drains it may possibly suggest contamination introduced from an outside source (Durham 2010).

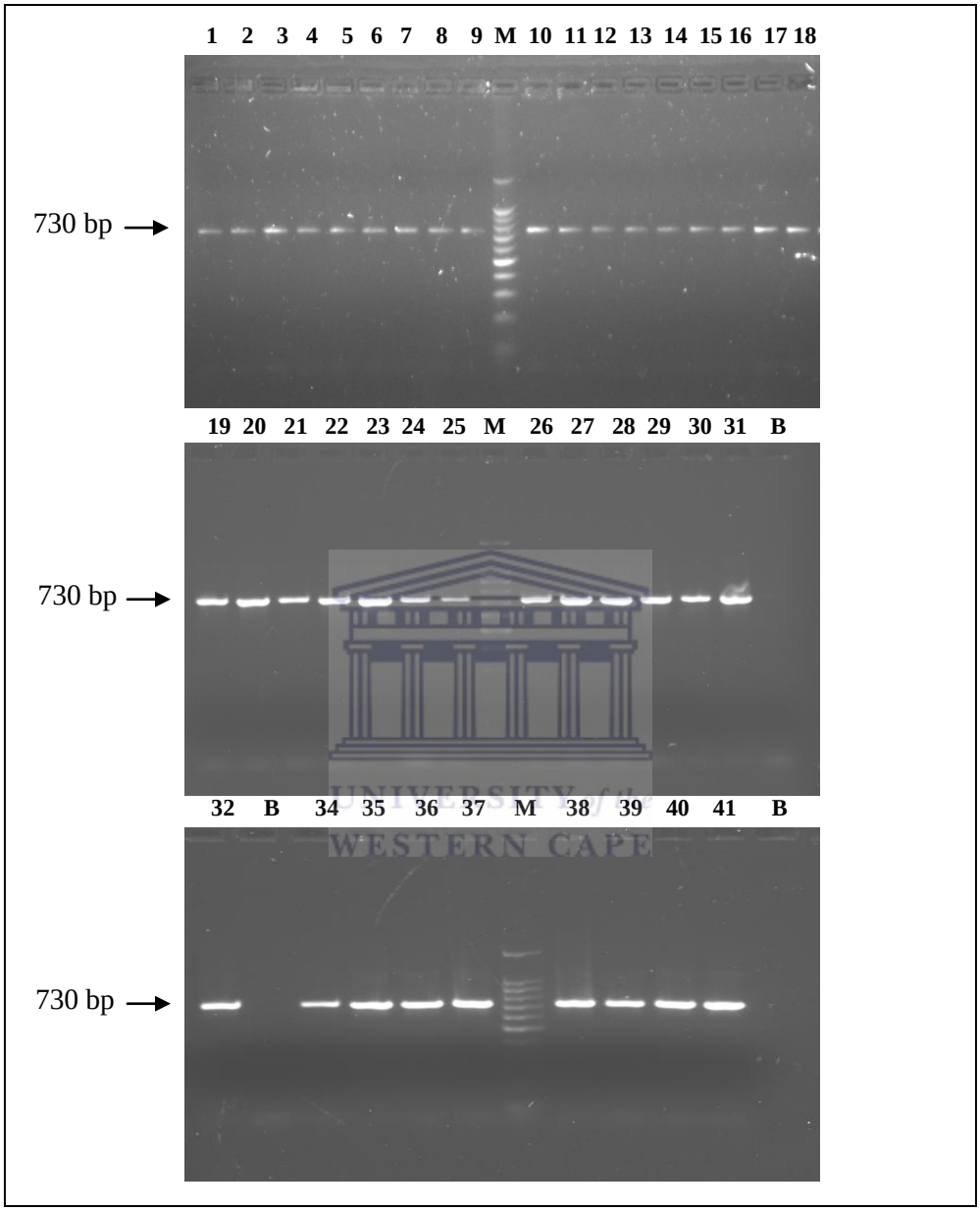


**Figure 5.2c** PCR amplification of the *hly* gene of *L. monocytogenes* from DNA isolated from meat, vegetables and salads and environmental samples. **M**: 100 bp DNA ladder (Promega); **B**: negative control (water); **lanes 1-17**: *L. monocytogenes* DNA isolated and amplified from meat samples; **lane 18**: positive control *L. monocytogenes* serotype 1/2c CIP 105.448; **lanes 19-31**: *L. monocytogenes* DNA isolated and amplified from 13 salad and vegetable samples; **lane 32**: positive control *L. monocytogenes* serotype 1/2c CIP 105.448; **lanes 34-41**: *L.*

The rooibos tea sample was acquired from a batch of tea leaves collected in a sterile 25 kg bag straight off the production line ready for distribution. The tea leaves had just undergone a steam pasteurization process through a 4.5m cylinder for 2.5 min at 160°C. The tea tested positive for *L. monocytogenes* on RAPID L. mono agar (blue colonies were observed) and PCR amplification of the *hly* gene (Figure 5.2d). *L. monocytogenes* is killed by pasteurization. The U.K Department of Health advised that RTE or similar foods should be exposed to a heat treatment of at least 2 min at 70°C whereas in the U.S, frozen dessert mixes require a process of 68.3°C for 30 min or 79.4°C for 25 s to ensure the destruction of *L. monocytogenes* (Curtis 2007; Dharmarha 2008) since ingredients such as fat, sugars and emulsifiers may protect *L. monocytogenes* from heat (Curtis 2007). In the processing stages for rooibos tea prior to the steam pasteurization step, there are no stages that would reduce the microbial load; instead temperature and other factors are such that micro-organisms could proliferate. The likelihood of a high microbial load present in the tea could be the reason for the high temperature employed for pasteurization. Prior to steam pasteurization of the rooibos tea, no *L. monocytogenes* was detected (results not shown). The presence of *L. monocytogenes* in the final product after steam pasteurization could be a result of bacteria entering the packaging and eventually the product from contaminated floors in the processing plant. No nets were used to cover the shoes of individuals entering the processing facility and consideration has to be given to the fact that the source of contamination was the floors of these establishments (contamination could have been introduced by the soil from the outside). Contamination

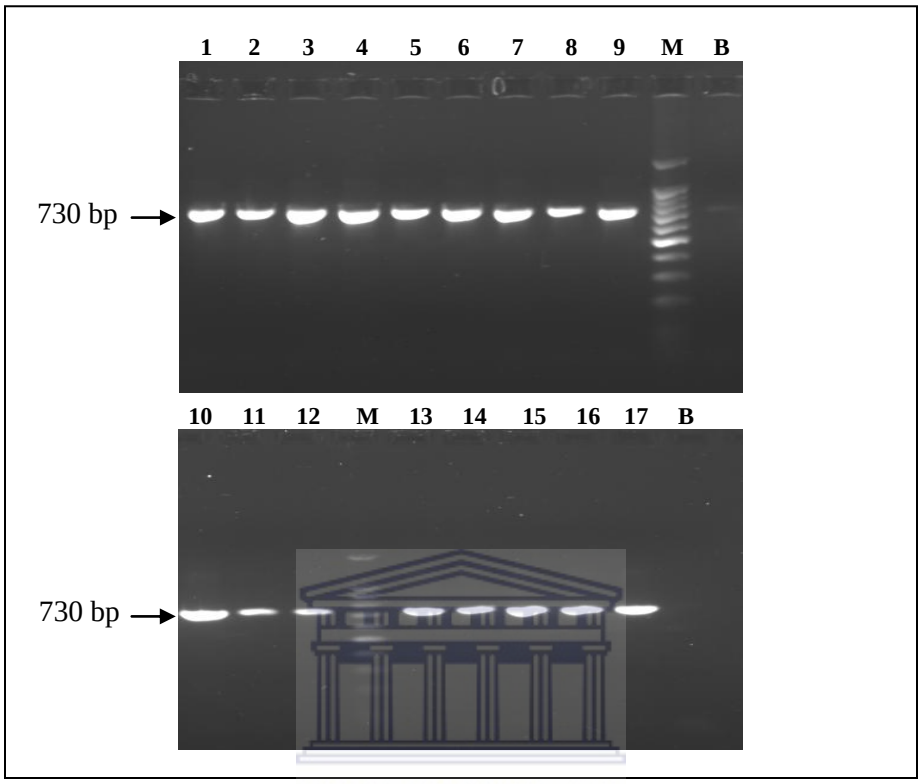




**Figure 5.2d** PCR amplification of the *hly* gene of *L. monocytogenes* from DNA isolated from environmental samples in the salad and vegetable food processing environment, seafood, rooibos tea, chicken blood and from water samples at water accumulation points in the avocado food processing plant. **M**: 100 bp DNA ladder (Promega); **B**: negative control (water); **lanes 1-5**: *L. monocytogenes* DNA isolated and amplified from five environmental samples in the salad and vegetable food processing environment; **lanes 6-8**: effluent rinse water from the guacamole food processing plant; **lane 9**: positive control *L. monocytogenes*; **lane 10**: *L. monocytogenes* DNA isolated and amplified from a milk sample; **lane 11**: *L. monocytogenes* DNA isolated and amplified from an unknown food sample (Food 25), **lane 12**: *L. monocytogenes* DNA isolated and amplified from rooibos tea; **lane 13**: *L. monocytogenes* DNA isolated and amplified from chicken blood; **lane 14**: *L. monocytogenes* DNA isolated and amplified from seafood (snoek mince); **lanes 15-16**: *L. monocytogenes* DNA isolated and amplified from seafood (trout terrine); **lane 17**: positive control *L. monocytogenes*.

can easily be introduced from the outside environment and the presence of *L. monocytogenes* could lead to biofilm production in the food processing environment (Ford 2010). Only one batch of rooibos tea (25 kg) was available for testing so a conclusion cannot be made with regard to whether there is a high incidence of *L. monocytogenes* in the tea as the sampling number from various batches has to be considered in order to make such an assessment.

The milk sample that underwent pasteurization and a UV treatment (1000 J $L^{-1}$) tested positive for *L. monocytogenes* by culture based methods and PCR amplification (Figure 5.2d). The presence of *L. monocytogenes* in milk based products can be attributed to the contamination of raw milk or to post-pasteurization contamination (Carminati *et al*. 2004). More often, post-pasteurization contamination of milk is credited to the contamination of raw milk coupled with improper pasturization (Fleming *et al*. 1985).

**Table 5.1 *L. monocytogenes* isolates from human clinical cases, food and environmental sources characterized into lineage groups by PCR-RFLP analysis**

| ISOLATE | PCR (*hly gene*) | RFLP ANALYSIS | | |
|---|---|---|---|---|
| | | Lineage I | Lineage II | Lineage III |
| **CLINICAL** | | | | |
| N = 7 | + | + | | |
| **FOOD / ENVIRONMENTAL/ BEVERAGE** | | | | |
| **GUACAMOLE** | | | | |
| Guacamole 1 | + | | + | |
| Guacamole 2 | + | | + | |
| Guacamole 3 | + | | + | |
| Guacamole 4 | + | | + | |
| Guacamole 5 | + | | + | |
| Guacamole 6 | + | | + | |
| Guacamole 7 | + | + | | |
| Guacamole 8 | + | | + | |
| Guacamole 9 | + | | + | |
| Guacamole 10 | + | | + | |
| Guacamole 11 | + | | + | |
| Guacamole 12 | + | | + | |
| Guacamole 13 | + | | + | |
| Guacamole 14 | + | | + | |
| Guacamole 15 | + | | + | |
| Guacamole 16 | + | | + | |
| Guacamole 17 | + | | + | |
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| Guacamole 27 | + | | + | |
| Guacamole 28 | + | | + | |
| Guacamole 29 | + | | + | |
| Guacamole 30 | + | | + | |
| Guacamole 31 | + | | + | |
| Guacamole 32 | + | | + | |
| Guacamole 33 | + | | + | |
| Guacamole 34 | + | | + | |
| Guacamole 35 | + | | + | |
| Guacamole 36 | + | | + | |

| ISOLATE | PCR (*hly gene*) | RFLP ANALYSIS | | |
|---|---|---|---|---|
| | | Lineage I | Lineage II | Lineage III |
| Guacamole 37 | + | | + | |
| Guacamole 38 | + | | + | |
| Guacamole 39 | + | | + | |
| Guacamole 40 | + | | + | |
| Guacamole 41 | + | | + | |
| Guacamole 42 | + | | + | |
| Guacamole 43 | + | | + | |
| Guacamole 44 | + | | + | |
| Guacamole 45 | + | | + | |
| Guacamole 46 | + | | + | |
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| Guacamole 77 | + | | + | |
| Guacamole 78 | + | | + | |
| Guacamole 79 | + | | + | |
| Guacamole 80 | + | | + | |
| Guacamole 81 | + | | + | |
| Guacamole 82 | + | | + | |
| Guacamole 83 | + | | + | |
| Guacamole 84 | + | | + | |

| ISOLATE | PCR (*hly gene*) | RFLP ANALYSIS | | |
|---|---|---|---|---|
| | | Lineage I | Lineage II | Lineage III |
| Guacamole 85 | + | | + | |
| Guacamole 86 | + | | + | |
| Guacamole 87 | + | | + | |
| Guacamole 88 | + | | + | |
| Guacamole 232 | + | | + | |
| Guacamole 233 | + | | + | |
| Guacamole 229b | + | | + | |
| Guacamole 221 | + | | + | |
| Guacamole 218 | + | | + | |
| Guacamole 241 | + | | + | |
| Guacamole 219 | + | | + | |
| Guacamole 223 | + | | + | |
| Guacamole 230a | + | | + | |
| Guacamole 245 | + | | + | |
| Guacamole 65.31 | + | | + | |
| Guacamole 239 | + | | + | |
| Guacamole 65.32 | + | | + | |
| Guacamole 231 | + | | + | |
| Guacamole 242 | + | | + | |
| Guacamole 243 | + | | + | |
| Guacamole 244 | + | | + | |
| Guacamole 235 | + | | + | |
| Guacamole 240 | + | | + | |
| Guacamole 223 | + | | + | |
| Guacamole 213a | + | | + | |
| Guacamole 214b | + | | + | |
| Guacamole 216 | + | | + | |
| Guacamole 209 | + | | + | |
| **VEGETABLES** | | | | |
| Carrot from coleslaw | + | + | | |
| Carrot | + | + | | |
| Coleslaw | + | + | | |
| Coleslaw | + | + | | |
| Coleslaw | + | + | | |
| Danish feta salad | + | + | | |
| Spring onion after dipping (peroxyacetic acid 150 ppm) | + | + | | |
| Spring onion | + | + | | |
| Spelt and legume salad – made with ‘stampkoring’ | + | + | | |
| Hulled barley salad (made with pearl barley) | + | + | | |
| Greek wheatberry salad (made with chickpeas) | + | + | | |
| Legume salad | + | + | | |
| Barley salad | + | + | | |

| ISOLATE | PCR (*hly gene*) | RFLP ANALYSIS | | |
|---|---|---|---|---|
| | | Lineage I | Lineage II | Lineage III |
| **SEAFOOD/FISH** | | | | |
| Snoek mince | + | + | | |
| Trout terrine | + | + | | |
| Trout terrine | + | + | | |
| **MEAT** | | | | |
| 1 sample | + | + | | |
| 16 samples | + | | + | |
| **ROOIBOS TEA** | | | | |
| 1 Sample | + | + | | |
| **MILK** | | | | |
| Milk (underwent UV treatment) | + | + | | |
| **ENVIRONMENTAL / OTHER** | | | | |
| **From food manufacturing plant of salads/vegetables** | | | | |
| Left soup filler (machine used to fill bags with soup) | + | + | | |
| Soup filler | + | + | | |
| Cabbage shredding machine | + | + | | |
| Kitchen drain | + | + | | |
| Kitchen drain | + | + | | |
| Kitchen drain | + | + | | |
| Kitchen drain | + | + | | |
| Spatula for scooping coleslaw | + | + | | |
| Spatula for scooping coleslaw | + | + | | |
| Mixed trough – trough used for mixing coleslaw in | + | + | | |
| Mixed trough | + | + | | |
| A swab sample from work in process (WIP) chiller water unit – a holding chiller for vegetables | + | + | | |
| WIP chiller water unit | + | + | | |
| **From food manufacturing plant of guacamole** | | | | |
| **Drain water** | | | | |
| Water accumulation points (avocado rinses) | + | | + | |
| Water accumulation points (avocado rinses) | + | | + | |
| Water accumulation points Drain 2a | + | | + | |
| **Other** | | | | |
| Food 25 | + | | + | |
| Chicken blood | + | | + | |

**Table 5.2** A representation of the percentage of the isolates grouped as lineage I, II and III

| | RFLP analysis | | |
|---|---|---|---|
| **Sample type and no. analyzed** | **Lineage I** | **Lineage II** | **Lineage III** |
| Clinical (n = 7) | n = 7 (100%) | - | - |
| **Total (n = 7)** | **n = 7 (100%)** | - | - |
| Guacamole (n = 112) | n = 2 | n = 110 | - |
| Vegetables and salads (n = 13) | n = 13 | - | - |
| Seafood (n = 3) | n = 3 | - | - |
| Meat (n = 17) | n = 1 | n = 16 | - |
| Rooibos tea (n = 1) | n = 1 | - | - |
| Milk (n = 1) | n = 1 | - | - |
| Chicken blood (n = 1) | - | n = 1 | - |
| **Total (n = 148)** | **n = 21 (14%)** | **n = 127 (86%)** | **n = 0** |
| Environmental (n = 16) | n = 13 | n = 3 | - |
| **Total (n = 16)** | **n = 13 (81%)** | **n = 3 (19%)** | **n = 0** |
| Other (Food 25) (n = 1) | - | n = 1 | - |
| **Total (food and environmental) (n = 165)** | **n = 34 (21%)** | **n = 131 (79%)** | **n = 0** |
| **Total ( food and environmental and clinical) (n = 172)** | **n = 41 (24%)** | **n = 131 (76%)** | **n = 0** |



Clinical samples (n = 7) were made available for molecular characterization. The information pertaining to the gender, age, clinical symptoms, underlying disease, death or recovery of patient and history of the patients' diet were not provided by the Hospital due to ethical consideration. However, it was confirmed that the patients' ages were in the very young or elderly range and they all had a compromised immune system. PCR amplification of the *hly* gene produced a 730 bp fragment for all seven isolates, indicative of a *L. monocytogenes* isolate. RFLP analysis with the three enzymes *Nde*I, *Hae*II and *Bsh*1285I for lineage group I, II and III respectively, characterized all clinical isolates as lineage I indicating that the serotype responsible for illness is either 1/2b, 3b, 4b, 4d, 4e or 7 (Figure 5.3). These results correspond with literature that suggest that lineage I

isolates are overrepresented in human listeriosis cases (Jinneman and Hill 2001; Ducey *et al*. 2007; den Bakker *et al*. 2008). Restriction digestion with enzyme *Nde*I, resulted in a banding profile of 410 bp and 320 bp which signified the presence of the SNP inherent to lineage I isolates. For lineage type II isolates; in the case where no SNP is present, the enzyme *Hae*II cuts all isolates resulting in 455 bp and 274 bp fragments (Figure 5.3). However; where the SNP for lineage II isolates are present, banding profiles of 278 bp, 274 bp and 178 bp are produced; which was not the result for the isolates from human clinical cases. Isolates were not digested with enzyme *Bsh*1285I, indicating that these strains do not belong to lineage type III.

Within lineage I, serotype 4b is particularly of concern because strains of this serotype contribute largely to the sporadic incidences of listeriosis outbreaks and epidemics worldwide. Also, it is more prevalent among clinical isolates compared to food isolates and is associated with a higher mortality rate (Ducey *et al*. 2007; Ward *et al*. 2010). Several genotyping data, generated as a result of various sub-typing tools, imply that of the serotypes commonly implicated in human listeriosis, serotype 4b is genetically rather homogenous and less diverse than the other serotypes; which is further supported by literature that report lineage I to be a genetically homogenous group (Tran and Kathariou 2002; den Bakker *et al*. 2008). However, only a small subpopulation of *L. monocytogenes* serotype 4b strains are implicated in the vast majority of listeriosis outbreaks which may be attributed to sequence variations in their virulence genes (Chen *et al*. 2005).

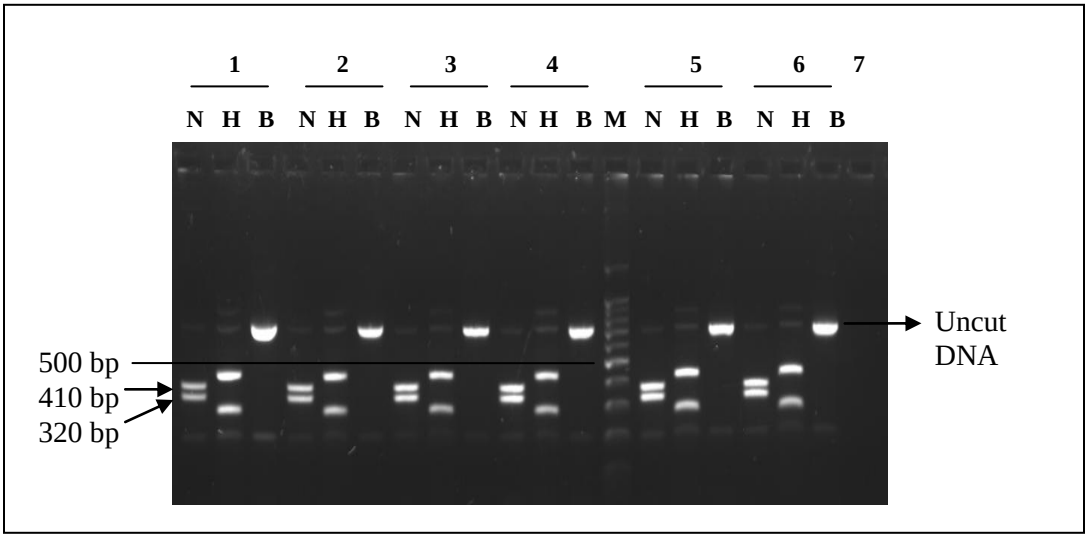



**Figure 5.3** RFLP analysis of *L. monocytogenes* DNA isolated and amplified by PCR. Enzymes *Nde*I (N), *Hae*II (H) and *Bsh*1285I (B) were used for lineage group classification of these isolates from human clinical cases. **M**: 100 bp DNA ladder (Promega); **1**: clinical isolate 1 digested with enzymes *Nde*I, *Hae*II and *Bsh*1285I; **2**: clinical isolate 2 digested with enzymes *Nde*I, *Hae*II and *Bsh*1285I; **3**: clinical isolate 3 digested with enzymes *Nde*I, HaeII and Bsh1285I; **4**: clinical isolate 4 digested with enzymes *NdeI*, *Hae*II and *Bsh*1285I; **5**: clinical isolate 5 digested with enzymes *Nde*I, *Hae*II and *Bsh*1285I; **6**: clinical isolate 6 digested with enzymes *Nde*I, *Hae*II and *Bsh*1285I; **lane 7**: negative control (water).

A total of 165 food and environmental samples were included in this study where 21% were identified as belonging to lineage I based on *hly* PCR-RFLP analysis and 79% were identified as belonging to lineage II (Table 5.2). From a total of 148 *L. monocytogenes* isolates enumerated from food samples; 14% were identified as belonging to lineage I and 86% were identified as belonging to lineage II. The isolates (n = 16) enumerated from the food processing environment and equipment were collected by swabbing in the food processing environment. These also included three effluent rinse water samples.

PCR-RFLP analysis characterized 81% of isolates as lineage I and 19% as lineage II. *L. monocytogenes* is ubiquitous in nature which accounts for the fact that it can be found in any food environment and explains its distribution in various food products, from fruit and vegetables to milk.

Of all the food samples collected, the sampling number was the greatest from the guacamole group. From the 112 samples of guacamole that were positive for *L. monocytogenes*, samples (n = 2) were characterized as lineage I and the remainder (n = 110) as lineage II (Figure 5.4a and Figure 5.4b). The high percentage of lineage II isolates in guacamole could be explained by the geographic specificity of strains in this lineage group to the guacamole food processing plant; it may be in-house contamination. The representation of isolates from a lineage group could however differ in food products in the same region. The fact that there is a representation of more than one lineage group could indicate cause for concern if proper control measures are not put in place. This implies that more than one *L. monocytogenes* serotype is present and the mere presence of this pathogen in this RTE food product is significant due to its ability to multiply to large numbers during refrigeration storage.

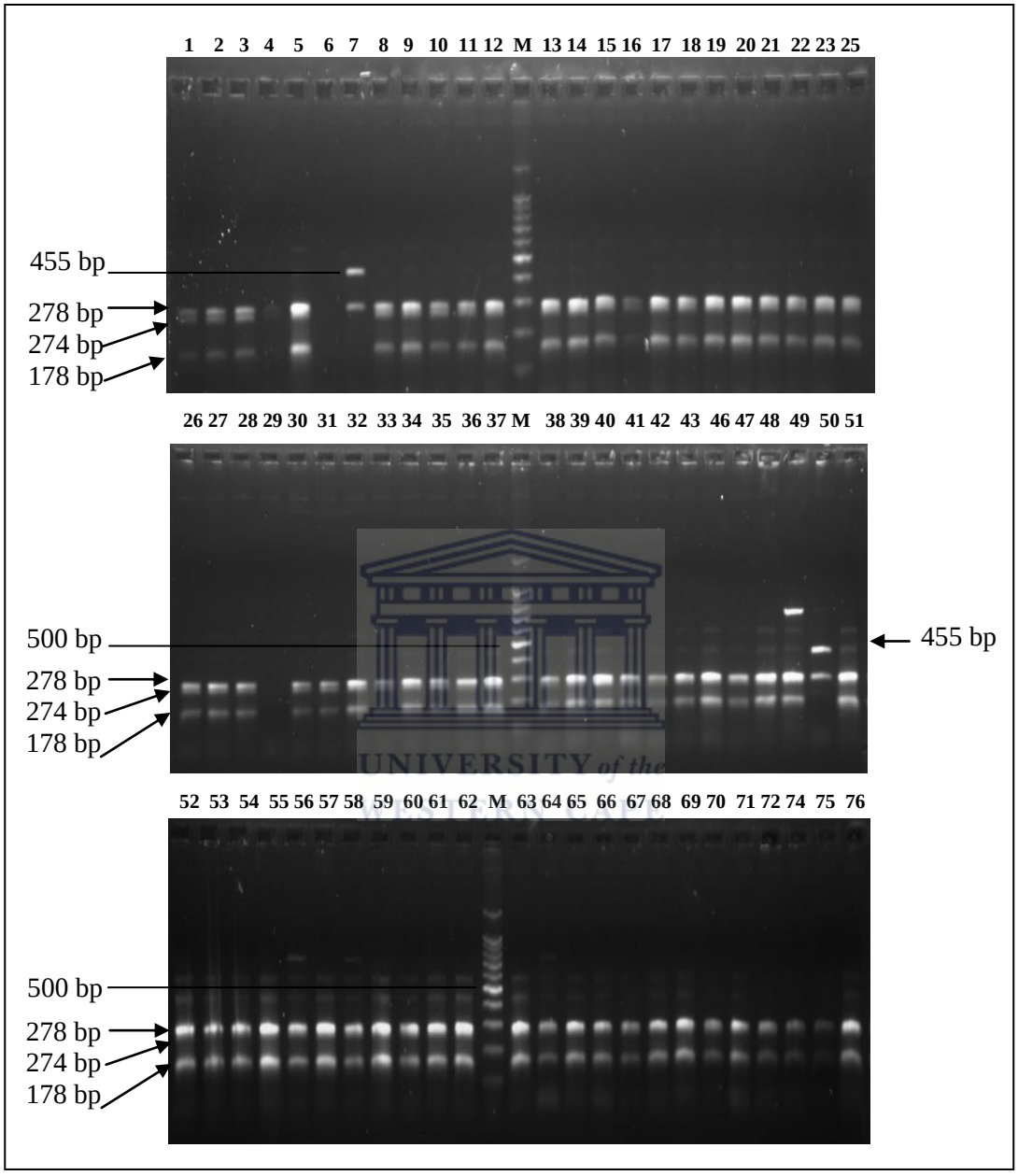



**Figure 5.4a** RFLP analysis of the *hly* gene of *L. monocytogenes* DNA isolated and amplified from guacamole samples. **M**: 100 bp DNA ladder (Promega); **lanes 1-76**: *L. monocytogenes* DNA from guacamole samples was digested with enzyme *Hae*II. All except isolate 7 and 50 were characterized as lineage II isolates. The DNA from isolate 6, 24, 29, 44, 45 and 73 was represented again in Figure 5.4b.

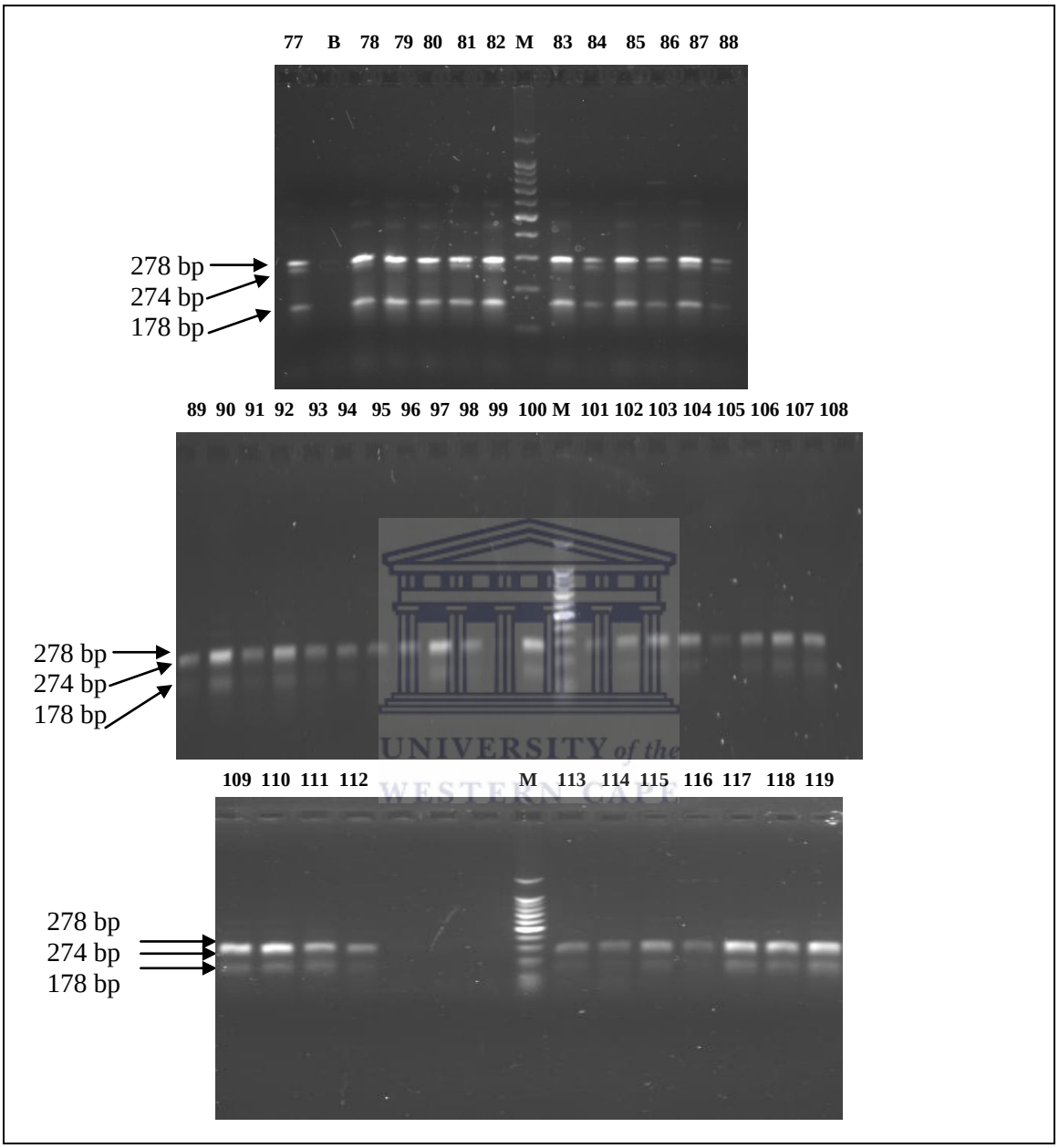



**Figure 5.4b** PCR-RFLP analysis of *L. monocytogenes* DNA isolated and amplified from guacamole samples. **M**: 100 bp DNA ladder (Promega); **B**: negative control (water); **lanes 77-112**: *L. monocytogenes* DNA from guacamole samples was digested with enzyme *Hae*II; **lanes 113-118**: *L. monocytogenes* DNA from guacamole samples 6, 24, 29, 44, 45 and 73 was digested with enzyme *Hae*II; **lane 119**: positive control *L. monocytogenes* serotype 1/2c CIP 105.448 (lineage II isolate).

The salad and vegetables samples (n = 13) included carrots, coleslaw, spring onion, Danish feta salad, spelt and legume salad and hulled barley salad. A total of three samples were collected from the sea-food manufacturing plant which included trout terrine and snoek mince. All the *L. monocytogenes* isolates enumerated from these samples were characterized as lineage I by PCR-RFLP analysis with *Nde*I (Figure 5.5a and Figure 5.5b). When the DNA of these isolates underwent RFLP analysis with enzymes *Hae*II and *Bsh*1285I, no positive results were obtained (data not shown).

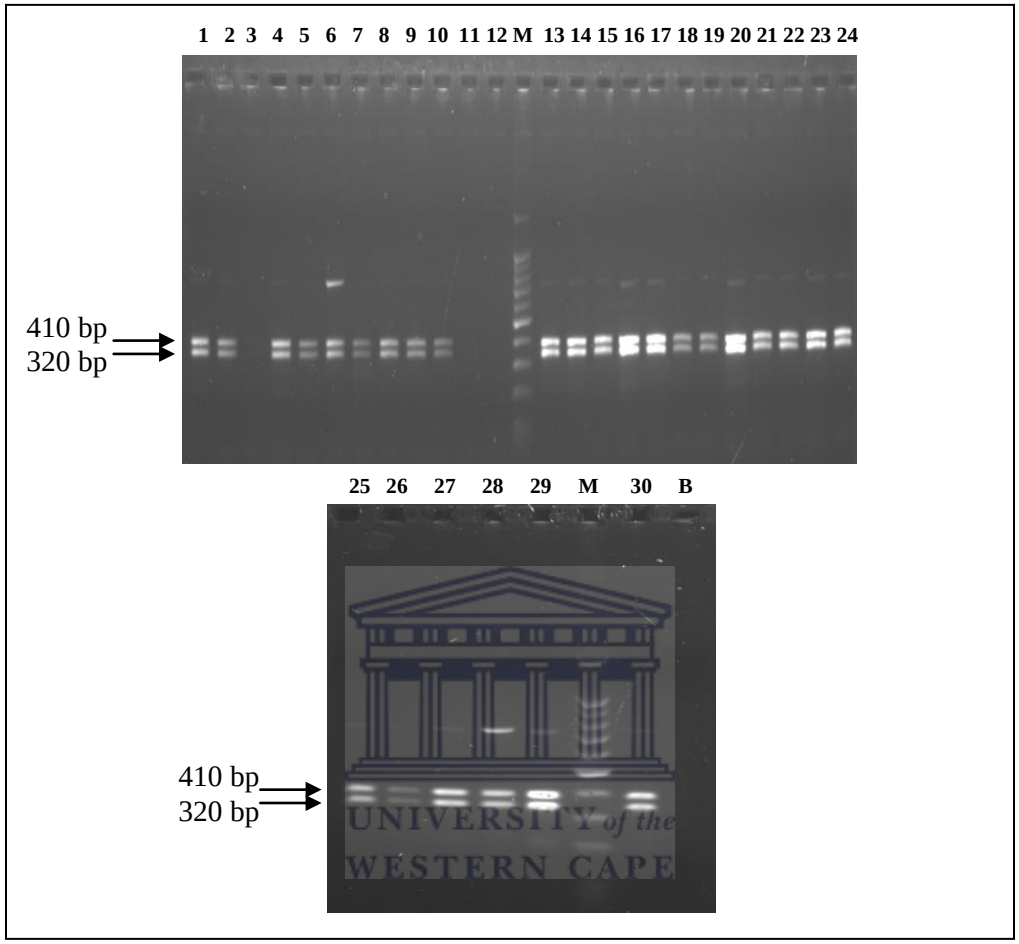



**Figure 5.5a** PCR-RFLP analysis of *L. monocytogenes* DNA isolated from salads and vegetables with enzyme *Nde*I. **M**: 100 bp DNA ladder (Promega); **B**: negative control (water); **lane 1**: carrot from coleslaw; **lane 2**: carrot; **lane 3**: coleslaw (rerun lane 25); **lane 4**: coleslaw; **lane 5**: coleslaw; **lane 6**: Danish feta salad; **lane 7**: spring onion after dipping in peroxyacetic acid; **lane 8**: spring onion; **lane 9**: spelt and legume salad; **lane 10**: hulled barley salad; **lane 11**: Greek wheatberry salad (rerun lane 26); **lane 12**: legume salad (rerun lane 27); **lane 13**: barley salad; **lane 14**: left soup filler; **lane 15**: soup filler; **lane 16**: cabbage shredding machine; **lane 17**: kitchen drain; **lane 18**: kitchen drain; **lane 19**: kitchen drain; **lane 20**: kitchen drain; **lane 21**: spatula for scooping coleslaw; **lane 22**: spatula for scooping coleslaw; **lane 23:** rooibos tea leaves; **lane 24**: milk; **lane 25**: coleslaw; **lane 26**: Greek wheatberry salad; **lane 27**: legume salad; **lane 28**: trout terrine; **lane 29**: trout terrine; **lane 30**: snoek mince.

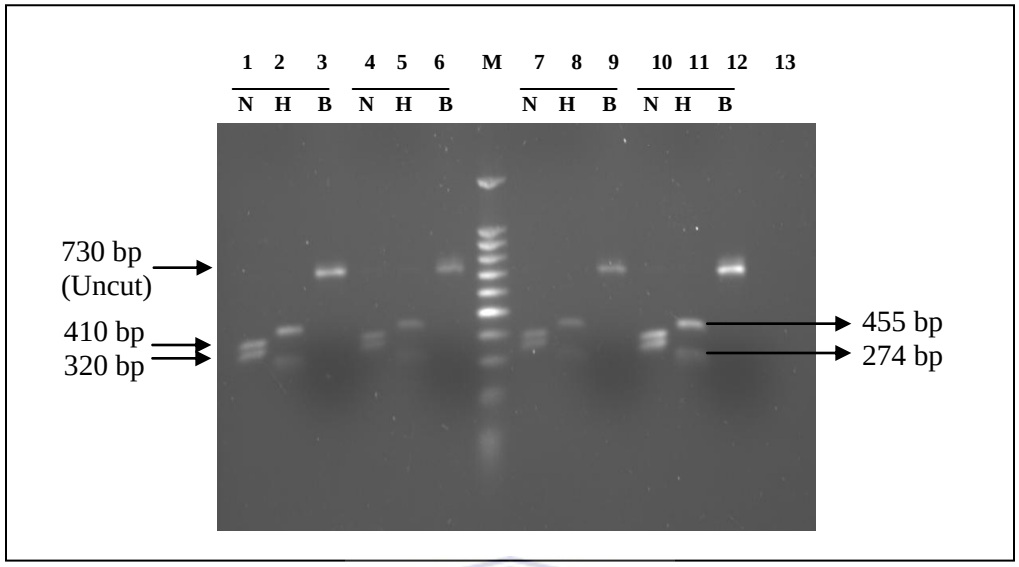



**Figure 5.5b** PCR-RFLP analysis of *L. monocytogenes* DNA isolated from the food processing environment where RTE salads and vegetables are prepared. Enzymes *NdeI* (N), *HaeII* (H) and *Bsh*1285I (B) were used lineage group identification. **M**: 100 bp DNA ladder (Promega); **lanes 1-3**: *L. monocytogenes* DNA isolated from a chiller water unit (a holding chiller for vegetables) digested with enzyme *Nde*I, *Hae*II and *Bsh*1285I respectively; **lanes 4-6**: *L. monocytogenes* DNA isolated from a chiller water unit digested with enzyme *Nde*I, *Hae*II and *Bsh*1285I respectively; **lanes 7-9**: *L. monocytogenes* DNA isolated from a mixed trough (trough used for mixing coleslaw in) digested with enzyme *Nde*I, *Hae*II and *Bsh*1285I respectively; **lanes 10-12**: *L. monocytogenes* DNA isolated from a mixed trough digested with enzyme *Nde*I, *Hae*II and *Bsh*1285I respectively.

PCR-RFLP analysis of *L. monocytogenes* isolates from an unknown food type from an outside laboratory (Food 25), chicken blood and water accumulation points (drains) for avocado rinses were all characterized as lineage type II by restriction digestion with enzyme *Hae*II (Figure 5.6). No restriction digest occurred with enzymes *Nde*I and *Bsh*1285I (data not shown).

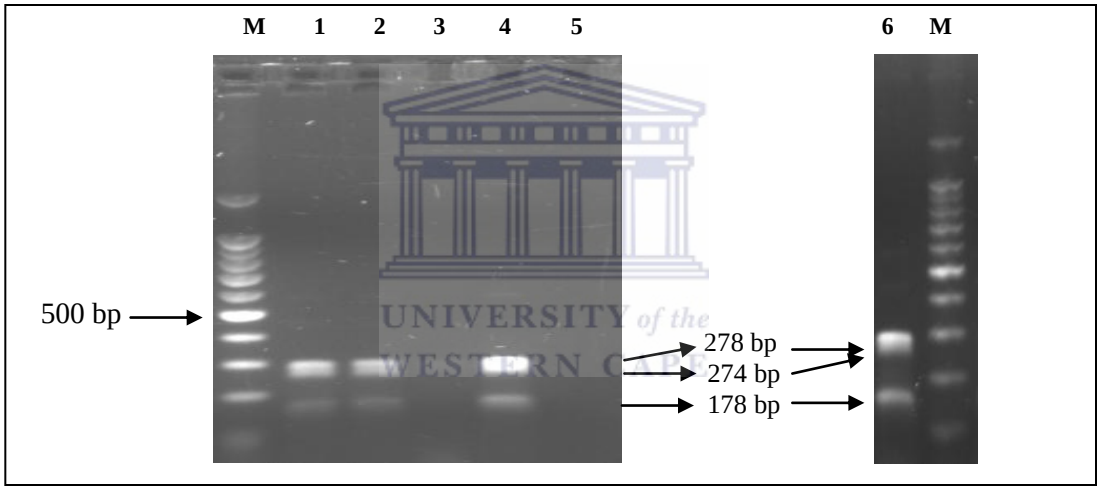



**Figure 5.6** PCR-RFLP analysis of *L. monocytogenes* DNA isolated from an unknown food type (Food 25), chicken blood and water accumulation points (drains) for avocado rinses. **M**: 100 bp DNA ladder (Promega); **lane 1**: unknown food type (Food 25); **lane 2**: chicken blood; **lane 3**: effluent rinse water (avocado); **lane 4**: effluent rinse water (avocado); **lane 5**: negative control (water) with enzyme *Hae*II; **lane 6**: effluent rinse water (avocado).

The consumption of meat products contaminated with *L. monocytogenes* has been linked to several outbreaks and deaths; which was observed already from the 1980s (Barros *et al*. 2007; Conly and Johnston 2008). *L. monocytogenes* isolates from meat were digested with enzymes *Nde*I, *Hae*II (Figure 5.7) and *Bsh*1285I. PCR-RFLP analysis characterized 16 of the isolates as lineage type II and one as lineage type I. No restriction digestion took place with enzyme *Bsh*1285I (data not shown). One *L. monocytogenes* isolate (no. 29) displayed no restriction digestion profile with enzyme *Hae*II, however RFLP analysis was redone for this sample and a digest profile positive for *Hae*II digestion was obtained (data not shown).

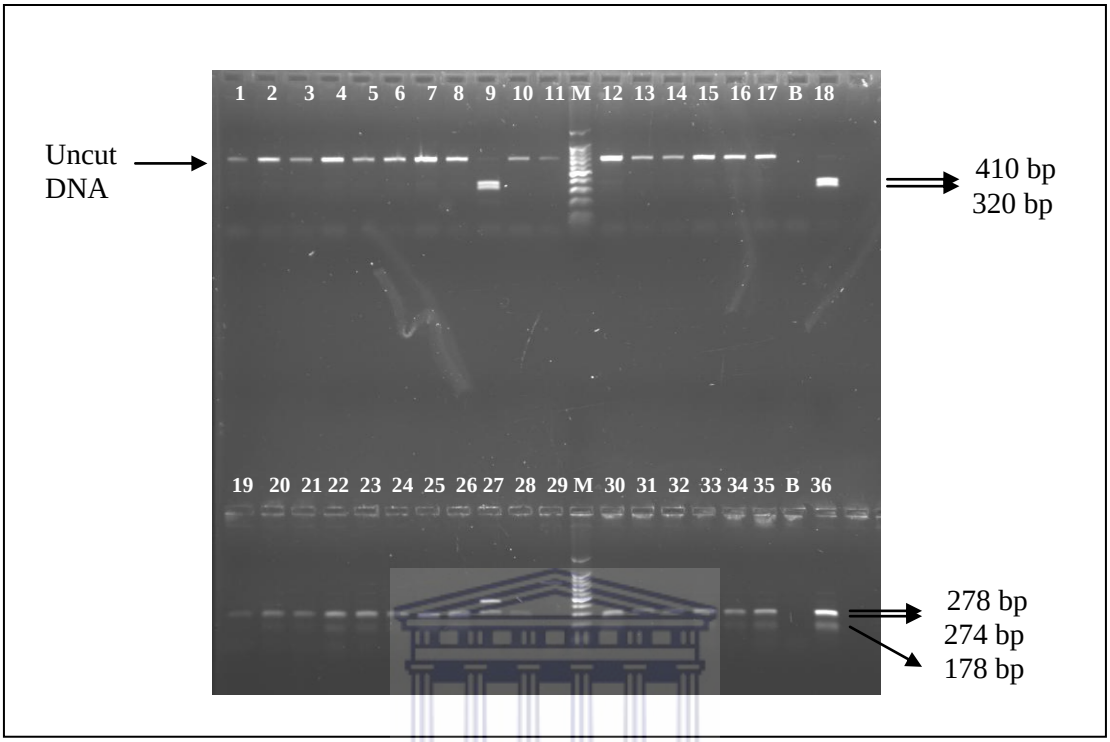





**Figure 5.7** PCR-RFLP analysis of *L. monocytogenes* DNA from meat samples. **M**: 100 bp DNA ladder (Promega); **B**: negative control (water); **lanes 1-17**: *L. monocytogenes* DNA isolated from various meat samples digested with enzyme *Nde*I, with only one isolate (lane 9) showing a digest profile; **lane 18**: positive control *L. monocytogenes* serotype 1/2b (CIP 105 449) lineage I isolate; **lanes 19-35**: the same isolates as represented by lanes 1-17 that underwent PCR-RFLP analysis with enzyme *Hae*II; **lane 36**: positive control *L. monocytogenes* serotype 1/2a (NCTC 7973) lineage II isolate.

To show the method of lineage group identification by PCR-RFLP analysis to be reliable and pertinent for future application, the MAMA-PCR technique was applied to the DNA isolated from some samples so that the results obtained by PCR-RFLPs would be legitimate and conclusive.

The MAMA-PCR technique was applied to *L. monocytogenes* DNA extracted from human clinical samples (n = 7). The results show that all seven isolates were amplified with primer set B and E which characterizes these isolates as lineage I. This method of lineage group identification corresponds with PCR-RFLP analysis which identified the human *L. monocytogenes* isolates as belonging to lineage I (Figure 5.8).

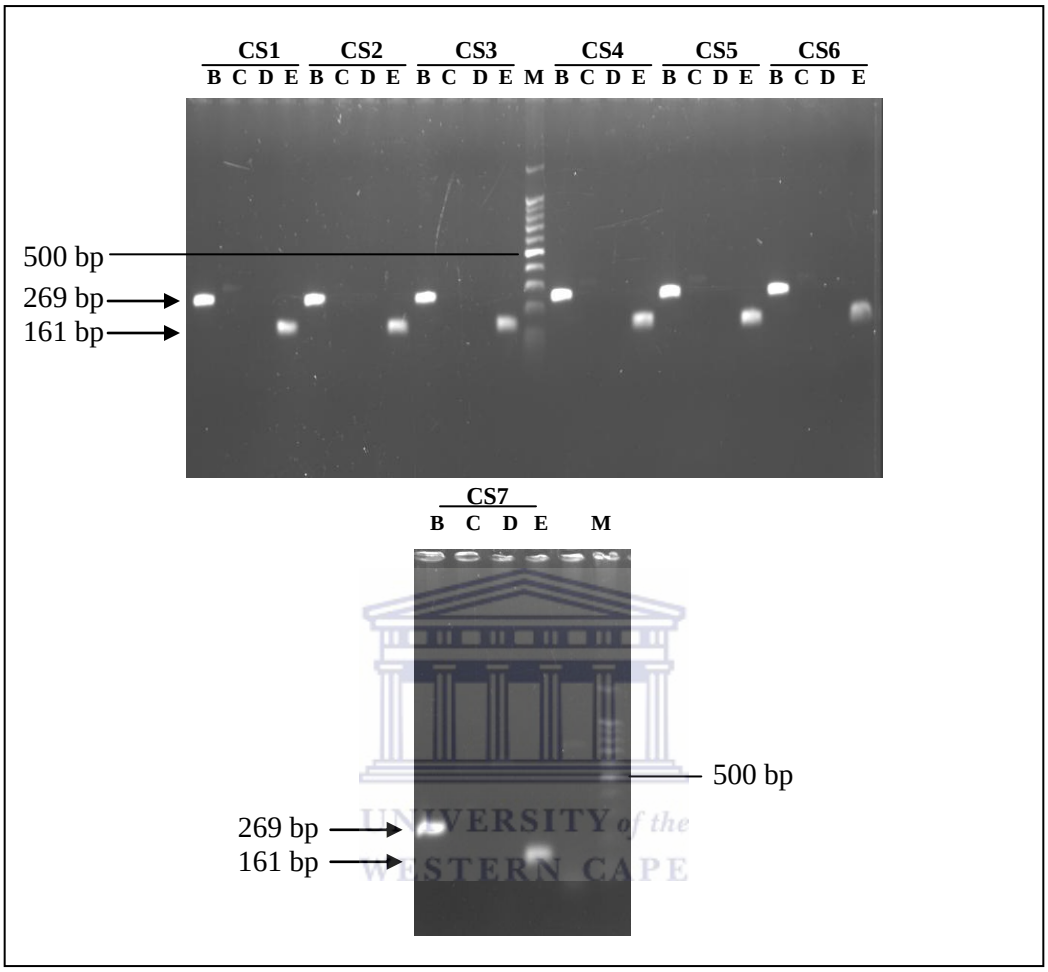



**Figure 5.8** MAMA-PCR of DNA extracted from cultures of seven human clinical samples (CS). **M**: 100 bp DNA ladder (Promega); **B**: MAMA-PCR primer set B; **C**: MAMA-PCR primer set C; **D**: MAMA-PCR primer set D; **E**: MAMA-PCR primer set E.

MAMA-PCR of DNA extracted from guacamole, salad and vegetables and samples from equipment and the processing environment was performed (Figure 5.9a and Figure 5.9b). Isolates from guacamole were characterized as lineage II subsequent to amplification by primer set D. Pertaining to the guacamole samples, faint DNA amplification took place with primer set E (specific for lineage type I isolates) (Figure 5.9a) but no amplification took place with primer set B, also specific for lineage type I isolates. However, characterization of isolates into lineage groups by PCR-RFLP analysis grouped all but two isolates as lineage type II. The final nucleotide (SNP) of the primer sequence in the 3' position of primer set E is specific only for lineage I isolates; there is also an intentional mismatch in the penultimate position. This faint amplification could however, be a result of the mismatch primer extending beyond the SNP and producing an amplicon specific for lineage I isolates.

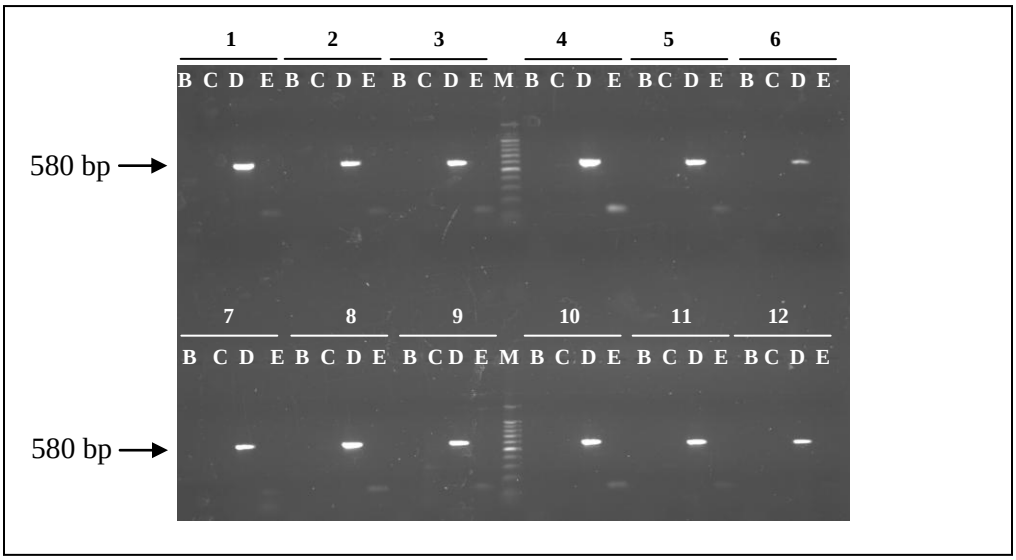



**Figure 5.9a** MAMA-PCR of *L. monocytogenes* DNA extracted from 12 guacamole samples. **M**: 100 bp DNA ladder (Promega); **B**: MAMA-PCR primer set B; **C**: MAMA-PCR primer set C; **D**: MAMA-PCR primer set D; **E**: MAMA-PCR primer set E. **Lane 1**: guacamole isolate 241; **lane 2**: guacamole isolate 219; **lane 3**: guacamole isolate 223; **lane 4**: guacamole isolate 230a; **lane 5**: guacamole isolate 245; **lane 6**: guacamole isolate 65.31; **lane 7**: guacamole isolate 65.32; **lane 8**: guacamole isolate 239; **lane 9**: guacamole isolate 230b; **lane 10**: guacamole isolate 231; **lane 11**: guacamole isolate 242; **lane 12**: guacamole isolate 243.

MAMA-PCR grouped all isolates from the salad and vegetables samples and the equipment and environment in that food processing plant as lineage type I (Figure 5.9b). PCR amplification occurred with primer sets B and E which are specific for isolates of this lineage group whereas no amplification took place when primer sets C and D were used. These results are in agreement with those obtained for PCR-RFLP analysis designed and employed in this study.

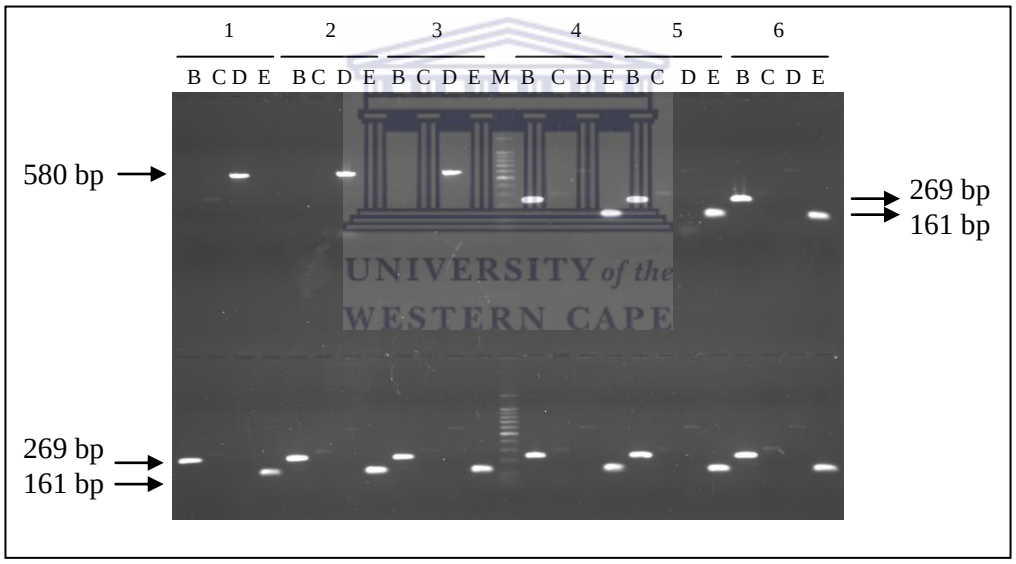



**Figure 5.9b** MAMA-PCR of *L. monocytogenes* DNA extracted from guacamole, salad and vegetables and environmental samples; where (1) refers to guacamole isolate 244 (2) guacamole isolate 235b (3) guacamole isolate 240 (4-12) refers to samples from the salad and vegetable food processing plant (4) kitchen drain (5) kitchen drain (6) soup filler (7) barley salad (8) legume salad (9) Greek wheatberry salad (10) cabbage shredding machine (11) kitchen drain (12) spatula for scooping coleslaw. **M**: 100 bp DNA ladder (Promega); **B**: MAMA-PCR primer set B; **C**: MAMA-PCR primer set C; **D**: MAMA-PCR primer set D; **E**: MAMA-PCR primer set E.

The DNA extracted from meat samples and amplified by MAMA primer sets were all characterized as lineage II isolates subsequent to amplification by primer set D (Figure 5.10a and Figure 5.10b). There was however, one isolate from meat that was amplified by primer set E and was identified as a lineage type I isolate (Figure 5.10 b). This is in accordance with the results from PCR-RFLP analysis which also identified this one isolate from meat as lineage type I and the rest as lineage type II.

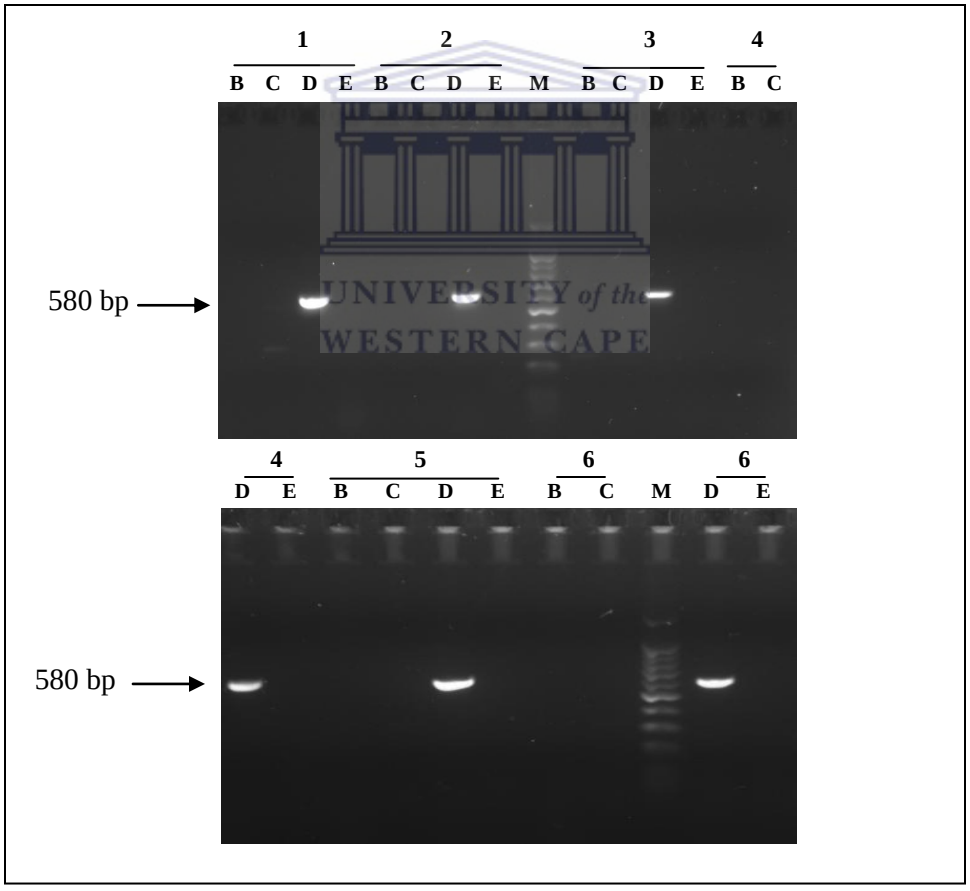



**Figure 5.10a** MAMA-PCR of *L. monocytogenes* DNA extracted from six meat samples which were all amplified with primer set D. **M**: 100 bp DNA ladder (Promega). **B**: MAMA-PCR primer set B; **C**: MAMA-PCR primer set C; **D**: MAMA-PCR primer set D; **E**: MAMA-PCR primer set.

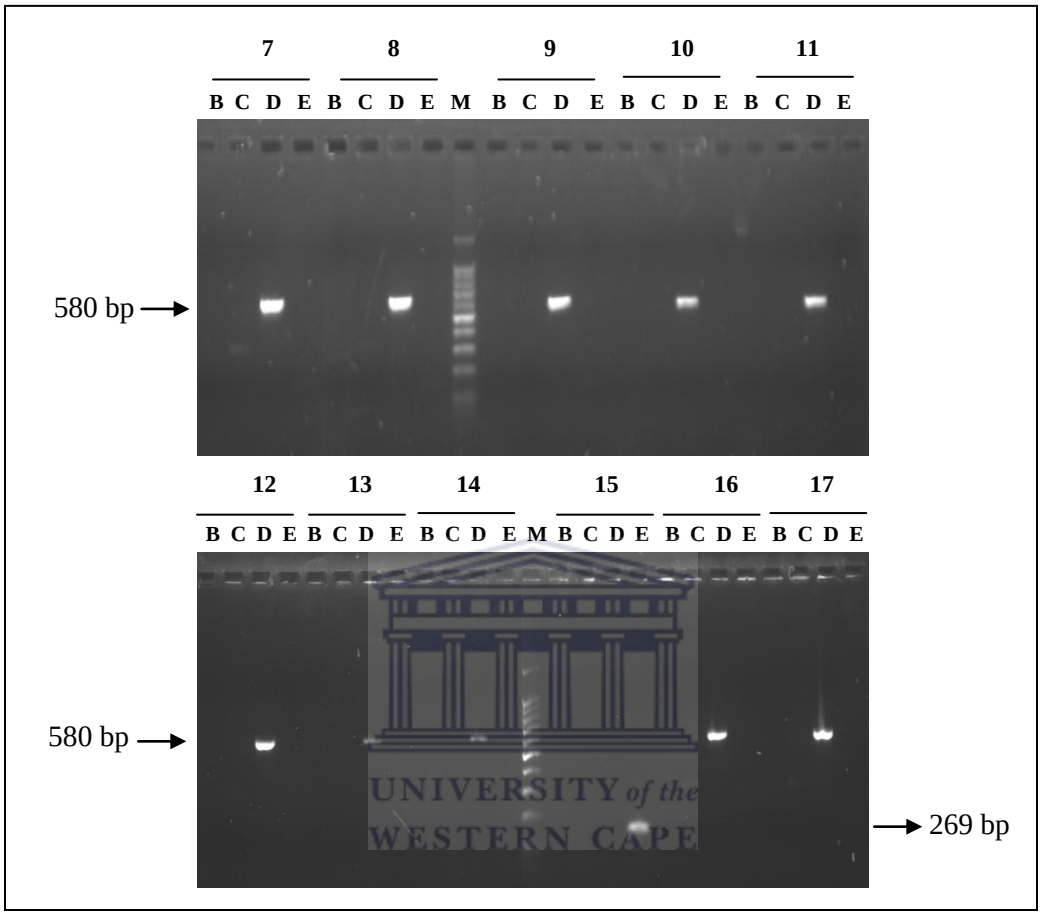



**Figure 5.10b** MAMA-PCR of *L. monocytogenes* DNA extracted from 11 meat samples. Samples (7-14, 16, 17) were all amplified with primer set D whereas sample 15 was amplified with primer set E. **M**: 100 bp DNA ladder (Promega); **B**: MAMA-PCR primer set B; **C**: MAMA-PCR primer set C; **D**: MAMA-PCR primer set D; **E**: MAMA-PCR primer set E.

Lineage III isolates are over-represented among isolates from food production animals (animal clinical cases) and under-represented among isolates from human clinical cases and foods (den Bakker *et al*. 2008). The low incidence of lineage III isolates in foods may explain their uncommon association with human listeriosis since most human listeriosis cases results from the ingestion of contaminated foods. Its minimal ability to cause disease in humans may be attributed to its limited capability to survive and multiply under stressful conditions commonly found in foods (Roberts *et al*. 2006). In this study, there was no representation of lineage III *L. monocytogenes* isolates among clinical or food samples, even in the chicken blood sample (chapter 6), which is in agreement with literature that report its absence in the food processing environment or human listeriosis cases.



Single *Listeria* isolates can spread in a food processing environment and establish itself as an endemic organism (Moorman *et al*. 2008). The *L. monocytogenes* isolates from the food processing environment obtained for this study appear to be predominantly lineage type II (with the largest contribution of isolates from the guacamole and meat samples). The appearance of one lineage group seems to predominate in or be confined to one food processing plant where there is not an even distribution or appearance of more than one lineage group. The over-representation of lineage II isolates is also a result of the sampling number; given that the number of guacamole samples accounted for 74% of the total food isolates. Isolates from lineage II could be suggestive of a persistent environmental contamination. Various molecular sub-typing data have shown that *L.*

*monocytogenes* can persist in food processing environments for very long periods of time (beyond 10 years) (Fugett *et al*. 2007; Rivoal *et al*. 2010).

Lineage I isolates were predominantly present from the salad and vegetables RTE food manufacturing plant and the sea-food processing plant next door. Only two isolates from the 112 guacamole samples were characterized as lineage I. All clinical isolates belonged to lineage I, which is in agreement with literature that show lineage I isolates to predominantly be responsible for human listeriosis, indicating a higher virulence potential in these strains. Lineage I isolates appear to be a genetically isolated population with a low recombination rate (den Bakker *et al*. 2008).



PCR-RFLP analysis designed and implemented in this study proved to be effective and efficient for the lineage group classification of isolates of *L. monocytogenes*. Results were comparable with that obtained by MAMA-PCR. The presence of the SNP conserved between lineage groups, means that the interpretation of PCR-RFLP results is consistent without ambiguity. Faint amplification could occur with MAMA-PCR for isolates that do not belong to a specific lineage group. Although intense amplification by primer sets do occur for isolates of a lineage group, fainter 'non-specific' bands may be indistinct and difficult or confusing to interpret when isolates of a different lineage group are weakly amplified by the mismatch/mutation primer. Five different primer combinations (including the diagnostic primer set) for MAMA-PCR are also used per isolate, making this technique tedious and repetitive.

The occurrence of *L. monocytogenes* in all the products tested can represent an important hazard since many of the food types were RTE foods that require no cooking and contamination can also be spread within the food processing plant through the equipment and utensils used in the preparation of the product (in the case of the RTE salads and vegetables). Possible cross-contamination could affect the safety of other products from the same food processing plant. The information collected can be used to take suitable steps in improving food safety as well as providing dietary advice to those who are at a greater risk for infection.

## 5.5 CONCLUSION

Given the important public health hazard posed by *L. monocytogenes* contamination of food and listeriosis infection, the ability to identify molecular fingerprint types is important in characterizing outbreaks. The variation of *L. monocytogenes* isolates associated with virulence, human illness and prevalence in foods makes molecular sub-typing tools for differentiating between serotypes important to improve future risk assessments and predict relative risks associated with serotypes. Molecular sub-typing provides information regarding genetic changes that affect the growth, pathogenic and virulence potential of *L. monocytogenes*.



The few human listeriosis isolates obtained for this study points to a low degree of listeriosis outbreaks or otherwise cases that are not reported. *L. monocytogenes* can be easily cultured from clinical specimens obtained from the placenta, blood or cerebrospinal fluid by directly plating on blood agar plates whereas food, environmental or stool specimens require selective enrichments for growth followed by plating on selective media. The results from this study indicate that the PCR-RFLP sub-typing approach designed for this study was effective and reproducible for lineage group distinction of *L. monocytogenes* serotypes.

The selection of an appropriate sub-typing method is on the basis of certain criteria that need to be met; some of which include, cost effectiveness, discriminatory ability,

reproducibility and ease of use. However, no one sub-typing method performs optimally for all criteria specified in every instance (Wiedmann 2002).

PCR-RFLP analysis determined that all clinical isolates were lineage type I and that isolates from food and environmental sources were characterized as lineage I (21%) and lineage II (79%). However, compared to various food types, the sampling number from guacamole was the largest and *L. monocytogenes* isolates from this food group were predominantly lineage type II. *L. monocytogenes* serotypes 1/2a and 1/2c are those mainly adapted to the food environment and are lineage type II isolates.

The surveillance of listeriosis around the world should be improved to have a better understanding of the burden of the disease and to prevent food-borne outbreaks. The data generated would provide sufficient information regarding the real impact of listeriosis in the population. This is important especially since the fatality rate with *L. monocytogenes* infection is higher than other food-borne pathogens.

Both avocados and guacamole can be contaminated by spoilage and pathogenic micro-organisms and moulds as a result of improper storage or proper storage beyond the expiration date. Since avocados and guacamole are perishable food products, they should be discarded when storage conditions are improper or when they have reached their expiry date. In the preparation and manufacturing of guacamole in a food processing

plant, adequate HACCP systems need to be implemented to eliminate the risks associated with *L. monocytogenes* contamination and to ensure health safety for the consumers.

It may be advisable to cut off one or two centimeters from the stem ends of the avocado as *L. monocytogenes* may enter there. The skin of the avocado may also be washed with soap and water to prevent the transference of *L. monocytogenes* to the interior or pulp of the avocado when cutting the fruit (Marler 2008).

Preventing listeriosis involves the implementation of adequate and effective quality assurance systems in the food industry as well as focusing on basic food safety for high risk groups of individuals. The implementation of HACCP procedures from the raw material to the finished product is important to reduce the incidence of contamination within the food-processing plant. Straightforward and essential hygiene principles that could reduce the risk of food-borne illness include, but are not limited to, adhering to the instructions on labels for adequate preparation and storage of food, maintaining a clean and proper functioning refrigerator, minimizing cross contamination where raw food may come in contact with RTE food or where knives and cutting boards are used for raw foods then not cleaned properly and re-used for RTE foods (Conly and Johnston 2008).



This research proves useful for practical and epidemiological purposes especially problems associated with food-borne listeriosis and the virulence properties of serotypes.

These findings will be quite significant as there is a lack of surveillance and data on the epidemiology of *L. monocytogenes* serotypes in the food supply in Sub-Saharan Africa.

# CHAPTER 6

## Isolation of *L. monocytogenes* from blood obtained from slaughtered chickens at two poultry processing plants

### 6.1 ABSTRACT

**Aims:** The presence of *L. monocytogenes* in chicken blood sourced from a total of 90 different organic 'free range' and battery chickens was investigated. RFLP analysis would characterize *L. monocytogenes* isolates into lineage groups.



**Materials and Methods:** Culture based methods, including enrichments in selective media and cultivation on TBA media (supplemented with horse blood) for the determination of haemolytic activity together with PCR were used for the detection and enumeration of *L. monocytogenes* from chicken blood. One *L. monocytogenes* isolate was recovered by culture based methods which was identified as a lineage II isolate by PCR-RFLP analysis.

**Conclusions:** PCR results and culture based protocols indicated that *L. monocytogenes* was present to a large extent in the chicken blood and that its presence in the blood is an indication of contamination to the bird and possibly to the consumer if proper storage and cooking procedures are not followed. *L. monocytogenes* was detected more frequently by

PCR than by cultural methods which could be an indication of non-viable cells of *L. monocytogenes* or viable but non-culturable cells.

**Significance and impact of study:** Outbreaks of listeriosis linked to poultry products and the poultry processing plant have been reported. This study intends to highlight the incidence of *L. monocytogenes* associated with chickens in the Western Cape region and the risk of infection associated with mishandling the raw poultry or improper/inadequate cooking.

## 6.2 INTRODUCTION

All members of the *Listeria* genus are ubiquitous in nature and may be isolated from farm animals and their environment which may be a vehicle of food contamination and illness for humans. As *L. monocytogenes* infect animals, they are transmitted back into the environment through the blood, milk and excrement of these animals (Akpolat *et al*. 2004). Farm animals that are asymptomatic carriers may shed the bacterium in their faeces (Esteban *et al*. 2009). Listeriosis has occurred in chickens (Kurazona *et al*. 2003) where symptoms that manifest may include leg tremors, depression and a circling motion (Cooper *et al*. 1992). According to a 21 month survey by scientists at the Agricultural Research Service, incoming poultry was the primary source of *L. monocytogenes* contamination in commercial chicken cooking plants (Durham 2010). Chickens are a food type consumed worldwide and can be prepared in various ways for many different types of dishes. Fully cooked, RTE products containing poultry meat have been implicated in large outbreaks of listeriosis (Capita *et al*. 2005). *L. monocytogenes* has also been isolated from raw poultry products purchased at retail outlets (Berrang *et al*. 2000) and another outbreak of encephalitic listeriosis in California broiler chickens were described (Cooper *et al*. 1992). In a study undertaken in Israel of various food types analyzed for *L. monocytogenes*, the highest prevalence was recorded in poultry (Vasilev *et al*. 2010). The pathogens found in chicken have been documented in many countries although the prevalence of different pathogens on South African poultry (carcasses) has not been researched well (van Nierop *et al*. 2005). However, various microbiological

research in South Africa on chicken carcasses showed that micro-organisms such as *E. coli*, *Campylobacter*, *Staphylococcus aureus*, *Listeria* species and *Salmonella* formed part of the chickens' gastrointestinal tract (Geornaras and von Holy, 2001; Geornaras *et al*. 2001; Bester and Essack, 2008).

In a study undertaken by van Nierop *et al*. (2005) on the contamination of chicken carcasses in the Guateng region, South Africa, they found that of 60 chicken carcasses examined for contamination with various pathogenic micro-organisms, a total of 19 (32%) was positive for *L. monocytogenes*. Both fresh and frozen chickens were tested and the results indicated that the numbers of *L. monocytogenes* detected from either type were not significantly different, again showing that this organism can grow at 4°C and survive freezer conditions.



Microbial pathogens have been known to be associated with chicken flesh and can cause illness or death in humans under certain circumstances; however practices such as refrigeration, preventing cross contamination and complete cooking may lessen the risk of contamination to consumers.

Battery chicken farming encompasses battery cages, a confinement for egg-laying hens (Pohle and Cheng 2009). It refers to conditions where the birds are housed or squeezed inside wire cages, deprived of exercise and food, debeaked to prevent their ability to eat and drink or attack each other (cannibalism); among other things. They are also fed

antibiotics to prevent viral and bacterial diseases and their feed is supplemented with vitamins and minerals to induce egg production. Antibiotic use may induce antibiotic resistance among food-borne bacteria that affect human health. They normally spend nine months in this confinement before being disposed of as a result of their unproductiveness. These cages or confinements are considered a breeding ground for bacteria which can be conveyed through the eggs of the battery hens to human consumers (International Vegetarian Union 2011). This practice is considered cruel and protested by many animal rights groups (Pohle and Cheng 2009). Battery cages are due to be banned in the European Union from 2012 whereas the use of battery cages are banned in countries such as Switzerland (the first country to do so), Belgium, Austria, Sweden and the Netherlands (Davis 2010). On the other hand, battery cage farming was said to have some benefits to the well being of the hens which include maintaining a small stable group size, low level of aggression and cannibalism (practice of debeaking) and high egg production (Pohle and Cheng 2009).



'Free range' chicken farming, on the other hand, is a method of farming whereby the birds are allowed to wander freely (outdoors) and are not enclosed in any manner. In this regard, feed costs are reduced, a higher quality animal or product is produced and this type of farming is considered more humane (Wang *et al*. 2009). The feed is usually not allowed to contain antibiotics (Husak *et al*. 2008). Different countries have different specifications for poultry or eggs to be called 'free-range'. The guidelines for raising organic chickens are more stringent though. Their feed is 95% organic and do not contain

antibiotics and are not supplemented with vitamins and minerals. Debeaking is an activity normally practiced on laying hens (not organic chickens) in order to reduce or inhibit aggressive feather pecking and cannibalism; however it causes much acute and chronic pain (Dennis *et al*. 2009).

Antibiotics fed to chickens may stimulate the growth of antibiotic resistant bacteria within the birds. These antibiotic resistant bacteria remain in the flesh and can be transmitted to humans through contact with the blood or the uncooked meat (Jones 2003). A blood or spinal fluid test is performed to determine if you have listeriosis. When treating bacterial infections in humans, antibiotics including ampicillin and penicillin are effective for the treatment of listeriosis. Where penicillin induces an allergic reaction in people, trimethoprim sulphamethoxazole is used for treatment (Brandriff 2003). *In vitro*, this organism was shown to be susceptible to penicillin, ampicillin, gentamicin, erythromycin, tetracycline, rifampicin and chloramphenicol and moderately susceptible to quinolones (Allerberger 2003). Cephalosporins and fluoroquinolones are not active against *L. monocytogenes*. Vancomycin has been efficient for *L. monocytogenes* bacteremia infection; however it does not cross the blood-brain barrier well enough for the effective treatment of meningitis (Koneman *et al*. 2006). Antibiotic therapy for human listeriosis should be administered for at least three weeks; however where HIV patients are concerned, antibiotic treatment may be lifelong to prevent recurring illness (Koneman *et al*. 2006).

Beta haemolysis (β-haemolysis) is sometimes referred to as complete haemolysis, which refers to complete lysis of red blood cells in the media around and under the colonies. The zone of haemolysis appears lightened and transparent. The enzyme produced by the bacteria, an exotoxin known as Streptolysin causes the complete lysis of red blood cells. β-haemolysis may be enhanced for some weakly β-haemolytic species when grown together with a strain of *Staphylococcus*. This is called the Christie Atkins Munch-Peterson (CAMP) test (Rodriguez *et al*. 1986; McKellar 1994)).

One of the factors associated with the virulence of *L. monocytogenes* is hemolysin, designated as listeriolysin O (LLO), without which this organism has been rendered avirulent due to the loss of haemolytic activity (Lachica 1996). Hemolysis is a key differentiating factor for *Listeria* spp. however there is not a stringent relationship between the amount of hemolysin produced and the virulence of the organism (Schuch *et al*. 1992; Lachica 1996). The phenotypic appearance of *L. monocytogenes* is similar to other *Listeria* spp, with the exception of haemolytic activity which aids in differentiating between the *Listeria* spp.

Haemolysis on blood agar is an important property in the detection and identification of *L. monocytogenes* (the only human and animal pathogen of the species) from the other non-pathogenic species of *Listeria*. The two species of *Listeria*, *L. monocytogenes* and *L. innocua* that tend to co-exist in the same environment, can be differentiated from one another in that *L. monocytogenes* is haemolytic and *L. innocua* is non-haemolytic, an important feature since the phenotypic characteristics of the two species are the same

(Beumer *et al*. 1996). Of the 6 species of *Listeria*, only *L. monocytogenes, L. ivanovii* and *L. seeligeri* show hemolytic activity on blood agar. The latter occurs less frequently in food but may be found in environmental samples, in which case further identification is required for distinction between this species and *L. monocytogenes* (Beumer *et al*. 1996). *L. monocytogenes* is weakly haemolytic on sheep or horse blood agar appearing as small, smooth, translucent colonies with a narrow zone of β-haemolysis (Žaloudíková *et al*. 2009). The haemolytic activity of *L. monocytogenes* may vary depending on the kind of medium or blood used; where the size of the haemolytic zone may be influenced by the blood agar base used (Fujisawa and Mori 1994). The zones of haemolysis produced by *L. monocytogenes* and *L. seeligeri* are less intense whereas *L. ivanovii* is considered strongly haemolytic (Bhunia 2008).



Pathogenic and non-pathogenic strains of *Listeria* are ubiquitous in nature and can be isolated from soil, vegetables and reservoirs as well as from healthy animals and humans (Kersting *et al*. 2010). The presence of the non-pathogenic species is often an indicator for the presence of much lower numbers of *L. monocytogenes* in food samples since *L. innocua* tends to outgrow *L. monocytogenes* in enrichment broths (Vaz-Velho *et al*. 2001; Willis *et al*. 2006). The failure to detect these low numbers may result in the proliferation of pathogenic *L. monocytogenes* during incorrect storage or refrigeration of foods. In this study, chicken blood samples were acquired from both organic 'free range' and battery chickens in order to determine whether the pathogenic *L. monocytogenes* was present and in turn identify which lineage groups are represented.

## 6.3 MATERIALS AND METHODS

### 6.3.1 Acquisition of chicken blood samples

Chicken blood samples were acquired from a chicken abattoir company in the Western Cape region of South Africa and received in sterile heparin (EDTA or lithium chloride) blood collection tubes. Each vial represented a blood sample of a chicken that was slaughtered. Blood was received in batches of 10 at a time. Samples were obtained from two farms that housed either organic or battery chickens and was stored at 4°C upon arriving in the laboratory.



### 6.3.2 DNA extraction

A blood sample (100 μl) was subjected to DNA extraction using either the GenElute blood genomic DNA kit (Sigma-Aldrich) or the ZR Insect / Tissue kit (ZymoResearch), following the manufacturers instructions.

### 6.3.3 PCR amplification and purification

For a 25 μl reaction, the mixture contained: 1 X PCR buffer (final concentration) (Bioline), $MgCl_2$ (final concentration 5 mM) (Bioline), dNTP's (final concentration 200 μM) (Roche Diagnostics), primers hlyF and hlyR (final concentration 0.3 μM each)

(Whitehead Scientific), 1U *Biotaq* DNA polymerase (Bioline), 1 μl template DNA ($10^0$). Amplification was carried out in a thermal cycler GeneAmp® PCR system 2700 (Applied Biosystems) with the following programme: Initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 40 s and a final extension step at 72°C for 5 min. The PCR products underwent electrophoresis on a 1.5% agarose D-1 LE gel (Whitehead Scientific) and visualized by staining with ethidium bromide. The amplified PCR products were viewed using the Alphaimager® HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC™ software version 4.0.0.

The 730 bp *hly* gene fragment was excised from the gel (following PCR and gel electrophoresis) and was purified using the QIAquick gel extraction kit (Qiagen).



### 6.3.4 RFLP analysis

The 730 bp *hly* PCR products were digested with the enzymes *Nde*I, *Hae*II and *Bsh*1285I for lineage group identification as described in section 5.3.4.

### 6.3.5 Selective enrichment and plating

For the samples that were positive by PCR for the amplification of *L. monocytogenes*, conventional enrichment steps were introduced into the protocol to determine whether the

*L. monocytogenes* was viable and culturable. A blood sample, 100 µl, was inoculated into 9 ml Listeria enrichment broth (LEB) media and incubated overnight at 35-37°C. Following this enrichment, 100 µl was inoculated into 9 ml ½ strength Fraser broth (FB) and incubated overnight at 37°C. A loopful of culture was then streaked onto chromogenic RAPID L. mono (Bio-Rad laboratories) for confirmation and identification of *Listeria* spp.

### 6.3.6 Haemolytic reaction on TBA media



To determine viability and culturability, the blood was also streaked onto tryptone blood agar (TBA) (Merck) media supplemented with horse blood (4%). The plates were incubated in gas jars under anaerobic conditions generated with gas sachets (gas generating kit; anaerobic system, Oxoid) at 37°C overnight after which the appearance of haemolysis was observed. The colonies were presumed positive for *L. monocytogenes* according to their appearance on TBA, namely; the size and colour of the colonies and the presence and types of haemolysis. The *L. monocytogenes* presumptive positive colonies were randomly selected based on this phenotypic appearance. For further confirmation, the suspect colonies were inoculated into 9 ml TSB and incubated at 37°C overnight, after which the DNA isolation procedure was performed (Agersborg *et al*. 1997) and PCR of the *hly* gene of *L. monocytogenes*.

## 6.4 RESULTS AND DISCUSSION

The blood samples underwent microbiological testing within two weeks to six months after collection. It was stored at refrigeration temperatures until analyzed. Some blood samples had coagulated upon arrival even though they were collected in anti-coagulation tubes and as a result these samples were refrigerated until use and streaked on TBA media for *Listeria* identification. Due to the fact that these blood samples had coagulated, they could not be subjected to DNA extraction using the kits. *L. monocytogenes* can survive and may even proliferate after storing in the refrigerator (Sommers *et al*. 2009). The ability to detect and enumerate *L. monocytogenes* after this time may be enhanced. Blood that had coagulated was directly streaked onto TBA to determine haemolytic activity.



A total of 90 chicken blood samples were analyzed of which (n = 20) were a representative of the battery chickens and (n = 70) from the organic 'free range' chickens. The samples (n = 20) from battery chickens all tested positive for the *hly* gene of *L. monocytogenes* by PCR. During the DNA extraction procedure using the blood genomic DNA kits, the blood would partly coagulate and not pass through the filter and some of the blood would block the filter, impairing the efficiency of the test. This could account for the presence 'background noise' as well as the shearing observed on the agarose gel since the filter was 'contaminated' (Figure 6.1A). The 'background noise' could also be attributed to serum proteins or erythrocyte debris (Cocolin *et al*. 1997).

The presence of non-specifics on the agarose gel hindered the progression of subsequent molecular sub-typing by RFLP analysis as a proper banding pattern for lineage group identification would be obscured.

The 730 bp fragment was excised from the agarose gel and purified using a QIAquick gel extraction kit (Qiagen) (Figure 6.1B). These 10 samples were representatives of blood from the next group of battery chickens. The purified DNA underwent electrophoresis on an agarose gel and a faint 730 bp fragment was the result. This was the result after repeating the experiment numerous times. Problems were encountered when performing RFLP analysis on these faint purified PCR products in that the DNA would degrade and there would be no digest profile for these samples. To increase the intensity of the purified product, PCR amplification was performed on these samples, however a 730 bp bold fragment was not the result but shearing. Attempts to overcome these problems included, diluting the amplified DNA ten-fold, reducing primer and dNTP concentrations for PCR; however, this was to no avail. Due to the time restrictions, it was not possible to proceed further with troubleshooting in order to advance with RFLP analysis. It appeared as though the DNA was lost during the purification process or the yield was very low. A possible explanation could be that it was trapped on the nylon membrane (QIAquick membrane) during the purification process; the DNA could then be eluted more times in order to attempt an increase in the yield of DNA. Nonetheless, the findings from this research concluded that *L. monocytogenes* is present and can be enumerated from chicken

blood which poses the need for adequate preventative measures for the risk of contaminations when storing and preparing poultry for consumption.

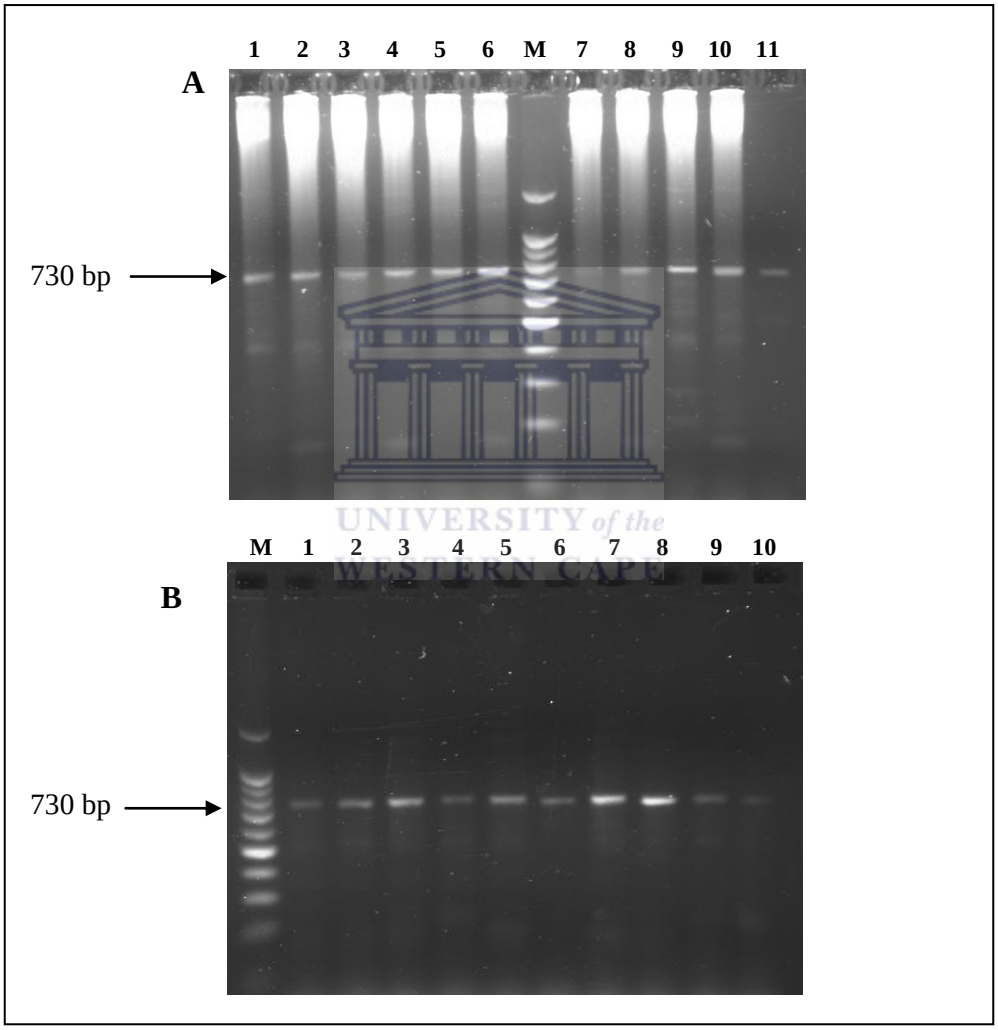




**Figure 6.1** PCR amplification of *L. monocytogenes* DNA extracted from battery chickens using the ZR Insect / Tissue kit (ZymoResearch). Blood samples were acquired from (**A**) 10 chickens and (**B**) 10 more chicken blood samples whose DNA was excised from the agarose gel and purified. **M**: 100 bp DNA ladder (Promega); **lane 11**: *L. monocytogenes* positive control serotype 4b (UWC L1).

Of a total of 70 blood samples acquired from free range birds, the genomic DNA from these samples (n = 30) was extracted by using the GenElute blood genomic DNA kit (Sigma-Aldrich). All 30 samples were positive for the *hly* gene of *L. monocytogenes* by PCR amplification. Of these PCR positive samples, 20 of the 30 samples are shown in Figure 6.2; for the other positives the data is not shown. Again, the same problems were encountered; non-specific bands were obtained and a single 730 bp fragment for the *hly* gene of *L. monocytogenes* was required for subsequent DNA analysis by RFLPs. The genomic DNA of the remaining 40 blood samples were not extracted using the DNA extraction kits; however they were tested for the presence of *L. monocytogenes* by culture based methods (Table 6.1).



**Table 6.1** *L. monocytogenes* enumerated from chicken blood by culture based methods and PCR

| | **Viability and haemolytic activity on 4% TBA** | **DNA extraction using a kit and PCR (*hly* gene) positive*** |
|---|---|---|
| **Organic/'free range' chickens** | 70 | 30 |
| **Battery chickens** | 20 | 20 |
| **Total** | 90 | 50 |

***** PCR was applied to DNA extracted directly from blood using a DNA extraction kit (non-culture based method).

Not all the samples were analyzed by PCR; instead only the numbers depicted in column three.

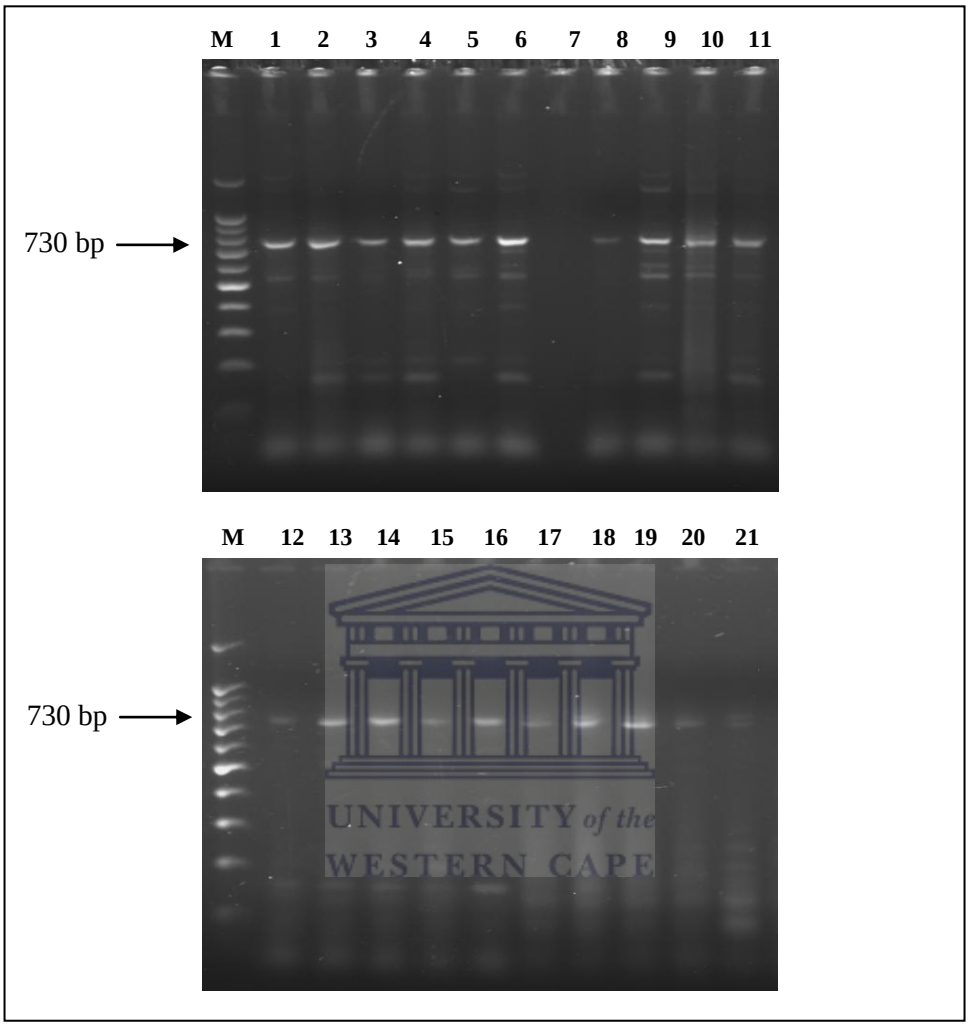
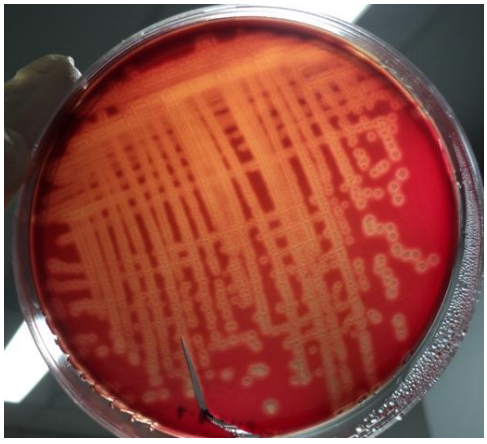


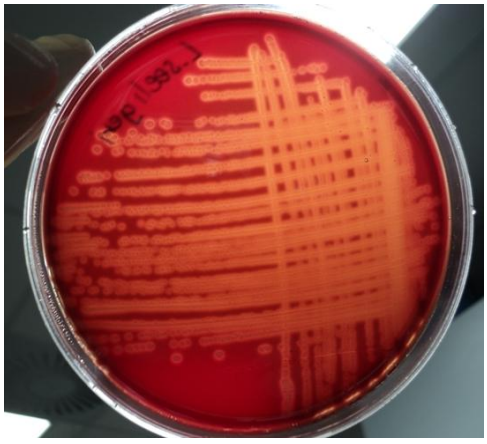

**Figure 6.2** PCR amplification of *L. monocytogenes* DNA extracted from free range chickens using the GenElute blood genomic DNA kit (Sigma-Aldrich). **M**: 100 bp DNA ladder (Promega); **lanes 1-21**: Blood samples of 20 chickens to which a DNA extraction method and PCR were performed; **lane 7**: no DNA loaded.

For comparison, pure cultures of all *Listeria* spp. were streaked onto TBA media to determine the interpretation of haemolysis on TBA media supplemented with 4% horse blood (Figure 6.3). *L. grayi*, *L. innocua* and *L. welshimeri* were non-haemolytic, appeared as small, grey colonies and were almost similar in their morphology and phenotypic appearance. *L. monocytogenes* serotype 1/2b (CIP 105 449), *L. seeligeri* and *L. ivanovii* all displayed haemolysis on this blood agar. Surprisingly, there was no distinction in their appearance on TBA media which could explain why the isolates from chicken blood that displayed haemolytic activity on TBA media were indistinguishable (Figure 6.4). Research undertaken by Mahmood *et al*. (2003) showed *L. monocytogenes* colonies to be small (1-2 mm in diameter), transparent with narrow zones of haemolysis that were described as being almost under the colony.

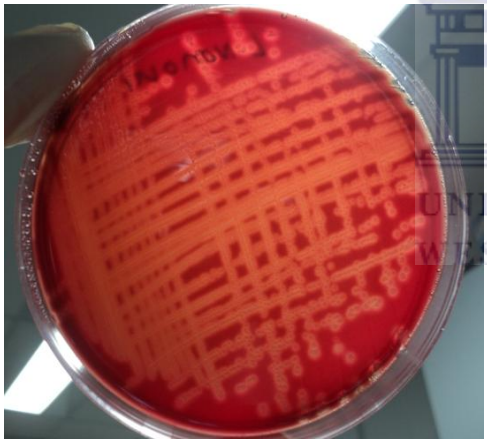
*L. monocytogenes*



*L. seeligeri*



*L. ivanovii*



*L. grayi*

*L. innocua*

*L. welshimeri*



**Figure 6.3** Pure cultures of *Listeria* species streaked onto TBA media.

**Figure 6.4** Chicken blood from free range chickens streaked onto TBA media.



The interpretation of haemolysis on blood agar may pose several problems since the haemolytic effect of *L. monocytogenes* has variable intensity where some strains display very weak haemolysis that may go unnoticed (Rodriguez *et al*. 1986; Schuch *et al*. 1992). With regard to haemolytic intensities, *L. monocytogenes* serotype 3a has been recognized as being especially haemolytic. Haemolysis is a key differentiating tool between *L. monocytogenes* and *L. innocua* since *L. innocua* is non-haemolytic whereas *L. monocytogenes* is weakly haemolytic (Schuch *et al*. 1992). When recovering *Listeria* spp. from selective enrichment broths in order to plate on selective media, the one problem that may occur is that the other haemolytic strains of *Listeria* may outgrow *L. monocytogenes* and increase the difficulty of differentiating between the haemolysis zones (Vaz-Velho *et al*. 2001). In this study, blood cultures were directly plated onto TBA media and after incubation, typical or presumptive positive *L. monocytogenes* colonies were selected as well as colonies that showed a different morphology typical of

other *Listeria* spp. All isolates that showed haemolytic activity were presumed to be either *L. monocytogenes*, *L. seeligeri* or *L. ivanovii*. Regardless of whether isolates were non haemolytic, these colonies were still included for PCR amplification of the *hly* gene of *L. monocytogenes*. Amplification of the *hly* gene would eliminate false negatives (as some *L. monocytogenes* strains were shown to be non haemolytic) and false positives (*L. seeligeri* and *L. ivanovii* that mimic the appearance of *L. monocytogenes*) (Fujisawa and Mori 1994; Beumer *et al*. 1996).

Of all the colonies randomly selected from TBA media, grown up in TSB and subsequently amplified by PCR, only one tested positive for the *hly* gene of *L. monocytogenes*, indicating that this *L. monocytogenes* isolate from chicken blood enumerated from a free range chicken was viable and culturable in this case (PCR positive result depicted in chapter 5). The inability to obtain positive PCR results after implementing culturing methods could be attributed to the fact that the interpretation of the haemolytic feature may be difficult, leading to the selection of non *L. monocytogenes* isolates from TBA media for molecular characterization. Also, the *L. monocytogenes* may be viable, but non-culturable. The failure to culture *L. monocytogenes* may be a result of injured cells in the blood or their failure to grow due to selective isolation conditions. However, the *L. monocytogenes* DNA that was extracted with the commercial DNA extraction kits and subsequently amplified by PCR may have been non-viable cells as PCR amplifies both viable and non-viable cells.

The use of microbiological methods that can detect all *Listeria* spp. is advantageous since the presence of non-pathogenic *Listeria* may signify a risk associated with the food-source indicating the possibility of low numbers of the pathogenic *L. monocytogenes* spp. that could perhaps be present. Once *L. monocytogenes* is in the poultry processing plant, it may become an inhabitant and may be able to survive sanitation procedures and become more widespread. Cross contamination of *L. monocytogenes* may be prevented in the poultry processing environment by the removing or replacement of processing equipment. There is a relatively high incidence of *L. monocytogenes* in poultry and as a result the contamination of processing equipment can also be high (López *et al*. 2008).



The DNA extracted from one *L. monocytogenes* isolate enumerated by culture based methods was amplified by PCR and subsequently digested with the enzymes *Nde*I, *Hae*II and *Bsh*1285I to determine their lineage grouping (Figure 6.5). The DNA was only digested by enzyme *Hae*II indicating a lineage II isolate. The DNA remained uncut when enzymes *Nde*I and *Bsh*1285I were used (data not shown).

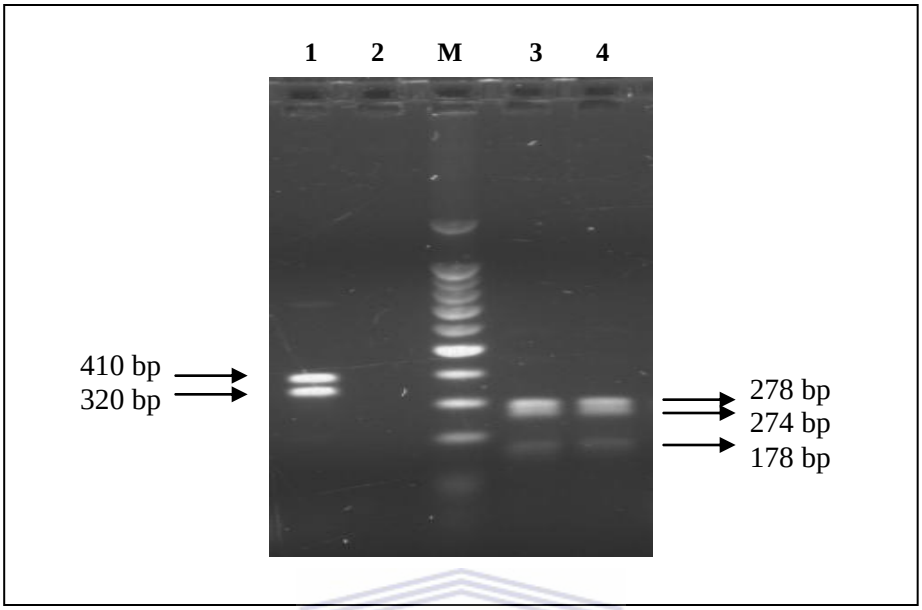



**Figure 6.5** RFLP analysis of *L. monocytogenes* DNA enumerated from chicken blood by culture based methods. **M**: 100 bp DNA ladder (Promega); **Lane 1**: *L. monocytogenes* lineage I isolate (serotype 1/2b) digested with enzyme *Nde*I; **lane 2**: negative control (water) digested with enzyme *Hae*II; **lane 3**: *L. moncytogenes* DNA from chicken blood digested with enzyme *Hae*II; **lane 4**: positive control *L. monocytogenes* serotype 1/2a NCTC 7973 (lineage II isolate) digested with enzyme *Hae*II.

The results of this study have demonstrated a high incidence of *L. monocytogenes* in chicken blood where 50 out of a total of 90 (56%) chicken blood samples tested positive for the *hly* gene of *L. monocytogenes* by PCR amplification. The remainder of the samples were not amplified by PCR but underwent culture based methods for the enumeration of *L. monocytogenes*. In other studies, where various food types were analyzed for *L. monocytogenes*, the highest prevalence was recorded in chickens, where the isolation rates were 18% (from a total of 100 raw whole chicken samples) and 27%

(in raw poultry) from another study (Akpolat *et al*. 2004; Vasilev *et al*. 2010). Results from various studies showed that the isolation rate of *L. monocytogenes* from chickens varied between 23% and 60% (Akpolat *et al*. 2004). Results from this study then indicate that the isolation rate of *L. monocytogenes* from the chicken blood samples was high. Where serotyping was performed in a study on the *L. monocytogenes* isolates from chickens, serotypes 1/2a, 1/2b, 1/2c, 3a, 4ab and 4b were isolated from 38.1% of domestic chicken meats and 30.5% of imported chicken meats from retail outlets in Japan (Kurazona *et al*. 2003). In the U.S., health officials recommend that all chicken flesh be treated as if it were contaminated as contamination with pathogens is so common (Jones 2003).

## 6.5 CONCLUSION

When chickens are slaughtered and processed, the unsanitary processes associated with mass production may expose the chicken flesh to faeces and chicken feed which may harbour many harmful bacteria including *L. monocytogenes*. The infected chicken blood may also be responsible for cross-contaminating other birds. The results from this study show that viable *L. monocytogenes* is present in chicken blood which may be an indicator for its presence on the flesh also.



The fact that *L. monocytogenes* can survive at refrigeration temperature illustrates its ability to survive and multiply on the final raw product stored at refrigeration temperatures. These findings show the importance of implementing proper storage and cooking procedures when preparing the chicken. Care must also be given to how raw chicken is stored in the refrigerator, taking into account chicken blood that may drip onto other RTE food also stored at refrigeration temperatures.

Haemolytic activity is a fundamental criterion for the differentiation of *Listeria* spp. however the interpretation of this feature may be difficult. Of all the samples, only one culture was positive for *L. monocytogenes* although molecular characterization showed that all DNA amplified by PCR was positive for the *hly* gene of *L. monocytogenes*. This may be explained as *L. monocytogenes* that is viable but non-culturable or it may simply be non-viable cells amplified by PCR. On the other hand, the failure to enumerate *L.*

*monocytogenes* by culture based methods does not conclude that it is in a non-viable state as it may have been non-*L. monocytogenes* colonies erroneously selected from TBA media or *L. monocytogenes* may have been viable but non-culturable in selective media or when outgrown by other *Listeria* spp.

Cooked and chilled chicken have been linked to sporadic cases of listeriosis and undercooked chicken were identified as risk factors for listeriosis (Rørvik *et al*. 2003). Preventative measures to avoid contamination of poultry products by *L. monocytogenes* must be implemented in the processing plants and the incidence of *L. monocytogenes* may be reduced by improving the hygiene. The severity of the human clinical case together with the high mortality rate, especially in those individuals with a compromised immune system emphasizes the critical importance of effective control measures to be implemented to limit this pathogen.

# CHAPTER 7

# Epidemiological typing of *Listeria monocytogenes* isolates from food and human clinical cases in sub-Saharan Africa by Pulsed Field Gel Electrophoresis

## 7.1 ABSTRACT

**Aim:** To examine the diversity of *Listeria monocytogenes* strains within and between food processing plants by applying PFGE.



**Materials and Methods**: A total of 102 *L. monocytogenes* isolates from food processing environments were included for PFGE analysis. PFGE of all the isolates were performed using the standard CDC PulseNet protocol. Pure cultures of *L. monocytogenes* were grown on TSA overnight. Bacterial culures were prepared by resuspending colonies in 1 X TE buffer. The cultures were embedded in 1% agarose plugs, lysed, washed and digested with *Apa*I. The DNA was digested in a CHEF-DR II system and gel images were viewed using the Alphaimager® HP system. PFGE patterns were then analyzed and compared using the BioNumerics version 6.5. Similarity clustering analysis was performed with BioNumerics by using the unweighted pair group-matching aligorithm (UPGMA) and the Dice correlation co-efficient with a tolerance of 1%.

**Conclusion**: Twenty-four pulsotypes were identified among the 102 strains analyzed. Isolates from human clinical samples were all closely related. One *L. monocytogenes* food isolate had the same pulsotype as the human clinical strain. This food product represents a potential health hazard.

**Significance and impact of study**: PFGE data are useful in outbreak investigations and to determine areas of cross-contamination. Information regarding in-house *L. monocytogenes* contamination is helpful to prevent finished product contamination. This is the first study dealing with the characterization of *L. monocytogenes* isolates from food processing environments and human clinical cases by PFGE in sub-Saharan Africa.

## 7.2 INTRODUCTION

Infections caused by *Listeria monocytogenes* require vigilance since it is important to recognize potential outbreaks very quickly (Graves and Swaminathan 2001). Pulsed Field Gel Electrophoresis (PFGE) is successfully used in epidemiologic investigations involving *L. monocytogenes* and other food-borne pathogens and is considered the 'gold standard' sub-typing method (Wiedman 2002; Ward *et al*. 2010). Since serotyping provides insufficient information in epidemiological investigations, highly discriminatory typing methods that correlate with serotyping are necessary. PFGE is considered a highly discriminatory and reproducible method for sub-typing *L. monocytogenes* (Graves and Swaminathan 2001; Yde and Genicot 2004; Chou and Wang 2006; Neves *et al*. 2008; Rivoal *et al*. 2010). PFGE classifies *L. monocytogenes* into subtypes or pulsotypes which in turn provides subtype discrimination regarded as the reference standard (Wiedmann 2002; Jeyaletchumi *et al*. 2010). DNA fragments produced by endonuclease digestion results in a banding profile or fingerprints that are useful when determining the degree of relatedness among isolates of the same species. Restriction enzymes are selected that cut the DNA infrequently to produce banding profiles consisting of eight to 25 bands. *AscI* and *Apa*I are the two enzymes most commonly used to produce DNA fragments in the range of 40-600 kb (Wiedmann 2002). The use of both enzymes in different reactions will improve the discrimination (Wiedmann 2002; Chou and Wang 2006)). The DNA fragments that are generated by PFGE are compared to determine the level of similarity or to differentiate between subtypes. Computer programmes such as Bionumerics

GelCompar software are used to determine if the strains under examination are distinguishable or indistinguishable or their level of relatedness (Koneman *et al*. 2006).

PulseNet is established in the US and comprises a network of public health and food regulatory laboratories that perform standardized PFGE sub-typing of food-borne pathogens (Graves and Swaminathan 2001; Hunter *et al*. 2005; Halpin *et al*. 2010). The exchange of PFGE patterns through PulseNet has shown much success in identifying and limiting food-borne listeriosis outbreaks. The value of PFGE for disease surveillance should increase as PulseNet expands internationally (Fugett *et al*. 2007).



PFGE types/isolates from food may be compared to isolates from human clinical cases in order to determine whether the strain from food is linked to human infection. On the other hand, since isolates from food and human cases may undergo mutations in their genetic material due to different environmental pressures (e.g. the human body), they may differ to some extent in their serotypes. As a result, the *L. monocytogenes* serotype from food responsible for the human illness may have a different serotype to the isolate from the human listeriosis case (Wiedmann 2002). According to Revazishvili *et al*. (2004), it is unlikely that a clear division between food and clinical isolates can be established since listeriosis in humans is mainly acquired through contaminated foods. The molecular sub-typing of bacterial isolates from human patients allows for the determination of specific genetic types responsible for clusters of human foodborne

disease cases. Furthermore, you can determine if they share a common ancestor (Wiedmann 2002).

Subtype discrimination by ribotyping showed that the subtypes common in foods in France and the US displayed a reduction in their competency to invade human intestinal epithelial Caco-2 cells. This was a result of mutations leading to premature stop codons (PMSCs) in the *inlA* gene. These mutations in *inlA* would explain the attenuated human virulence by these strains. *InlA* with premature stop codons are represented among much food isolates which supports why many food isolates show reduced virulence (Oliver *et al*. 2007). Attenuated human virulence, but to a lesser degree, may occur as a result of mutations in other virulent genes such as *hly* and *prfA* (Oliver *et al*. 2007). At least 14 mutations leading to PMSCs have been identified in *inlA* (Ward *et al*. 2010). Most food isolates are characterized as lineage II strains and strains of this lineage appear less likely to cause human disease compared to lineage I strains (Oliver *et al*. 2007). *L. monocytogenes* strains that have PMSCs in their *inlA* gene have reduced invasion efficiency in human intestinal epithelial cells and virulence attenuated phenotypes in animal models (Ward *et al*. 2010).



The aim of this research was to use PFGE to examine the diversity of *L. monocytogenes* strains in the food processing environment and the human isolates under investigation.

## 7.3 MATERIALS AND METHODS

### 7.3.1 Preparation of PFGE plugs from bacterial growth on agar

PFGE was performed according to a protocol by US PulseNet (a national network of public health laboratories that fingerprint foodborne pathogens) (Graves and Swaminathan 2001; CDC PulseNet 2009) with a few amendments to the protocol. PFGE was performed on 102 *L. monocytogenes* isolates (Table 7.1). Since all the guacamole isolates were received from one food processing plant and from the 112 isolates, 110 were characterized as lineage type II, close to 50% of the total number was selected for PFGE analysis which included the two isolates characterized as lineage type I.



Pure *L. monocytogenes* isolates enumerated from various ecosystems were streaked onto TSA and incubated overnight at 37°C. A sterile cotton swab (Lasec, SA) moistened with 1 X TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) was used to remove the bacterial growth which was resuspended in 2 ml 1 X TE buffer. Cells were gently resuspended in the buffer with the cotton swab with gentle agitation and no vortexing. The cell concentration was adjusted to an absorbance (optical density) of 1.00 at a wavelength of 610 nm on the Novaspec II (spectrophotometer) (Pharmacia BioTech) by either adding more cells or further diluting the sample. A volume of 400 µl of this cell suspension was transferred to a sterile eppendorf tube, to which 20 µl of Lyzozyme (Merck) (20 mg $ml^{-1}$) was added and mixed gently before incubating at 56°C in a shaking incubator (Zhicheng

Analytical Instruments) set at 60 rpm for 15 min. Thereafter, 20 µl Proteinase K (Roche Diagnostics) (20 mg $ml^{-1}$) was added to each suspension and mixed gently by inversion. Melted SeaKem Gold agarose (Lonza, Whitehead Scientific) (1%) was maintained at a temperature of 56°C and 400 µl was added to the bacterial suspension which was immediately dispended into a DR II reusable sample plug mold (Bio-Rad laboratories) in which to solidify (CDC PulseNet 2009).

### 7.3.2 Cell lysis in agarose plugs



Cell lysis buffer was prepared that included the following (final concentration): 50 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 1% sarcosine (N-Lauroylsarcosine, sodium salt Sigma-Aldrich) and 0.1 mg $ml^{-1}$ Proteinase K (Roche Diagnostics). Cell lysis buffer (5 ml) was required per plug wash and was transferred to a 15 ml Greiner tube (Lasec, SA) into which the agarose plug was transferred and lysed at 54°C for 2 h in a shaking incubator (Zhicheng Analytical Instruments) with vigorous agitation at 160 rpm (CDC PulseNet 2009).

### 7.33 Washing of agarose plugs following cell lysis

The lysis buffer was carefully removed including any liquid in the top of the greiner tube. Enough sterile distilled water and 1 X TE buffer was pre-heated to 50°C so that the plugs could be washed twice with 10 ml sterile distilled water and four times with 10 ml 1 X

TE buffer for 10 min each. Once the last wash was completed, the plugs were stored in 5 ml 1 X TE buffer at 4°C until restriction digestion was performed (CDC PulseNet 2009).

### 7.3.4 Restriction digestion of DNA embedded in agarose plugs

#### 7.3.4.1 Pre-restriction incubation step

The DNA plug was removed from the TE buffer and a sterile blade was used to slice a portion of the plug to be used for restriction digestion. The shape and size of the plug corresponds to the width of the bottom of the comb teeth. The plug was treated with a pre-restriction incubation step in a final volume of 200 μl with 1 X FastDigest restriction buffer (final concentration) (Fermentas) and sterile distilled water. The samples were incubated at 25°C for 10 min (CDC PulseNet 2009).



#### 7.3.4.2 Restriction digestion

After incubation the buffer was carefully removed with a pipette taking care not to damage the plug slice. Intact, high molecular weight DNA in plug slices, were digested with endonuclease *Apa*I (FastDigest) (Fermentas). For a 200 μl reaction per sample, the mixture contained: 1 X FastDigest restriction buffer (final concentration) (Fermentas), BSA (final concentration 0.1 mg/ml) (Whitehead Scientific), *Apa*I (FastDigest) (5 U $\mu l^{-1}$

per plug) (Fermentas). The sample was incubated at 25°C for 25 min (adapted from CDC PulseNet 2009).

#### 7.3.4.3 Casting agarose gel

Following the restriction digestion with *Apa*I, the enzyme/buffer mixture was removed with a pipette and 200 µl 0.5 X Tris-borate-EDTA (TBE) (prepared from 10 X TBE) was added to the plug and incubated at room temperature for 5 min. The plugs were then removed from the buffer and loaded onto the bottom of the teeth of the comb (which was placed in a horizontal position) and left to air dry for approximately one min. The gel comb was then positioned into the gel form/tray and 100 ml of 1% SeaKem Gold agarose (Lonza, WhiteHead Scientific), maintained at 55-60°C, was poured into the gel tray and left to solidify for approximately 30 min (CDC PulseNet 2009).



#### 7.3.4.4 Electrophoresis conditions

The DNA fragments underwent electrophoresis in the CHEF-DR II system (Bio-Rad laboratories) with 0.5 X TBE. Electrophoretic parameters were as follows: The initial switch time 4.0 s, final switch time 40.0 s, voltage 4.0 V $cm^{-1}$ and run time 22 h. The flow rate of the pump was set at approximately 0.75 l $min^{-1}$ (corresponds to setting the dial at 70) (adapted from CDC PulseNet 2009).

In the absence of the mini chiller (a refrigerated apparatus that maintains a buffer temperature of approximately 14°C in the electrophoresis cell chamber), the Tygon tubing was coiled into an ice bucket and furthermore the CHEF-DR II apparatus was set up in a room where the temperature was set between 5-7°C.

**7.3.4.5 Staining and analysis**

Once the run was completed, the gel was stained in an ethidium bromide solution (10 mg $ml^{-1}$) for 30 min and thereafter destained in distilled water for a total of 20 min (changing the water once after the 10 min) (adapted from CDC PulseNet 2009). The gel images were viewed using the Alphaimager® HP system (AlphaInnotech Corporation). Gel pictures were acquired using the AlphaEase FC™ software version 4.0.0. Gels were normalized by alignment with the *L. monocytogenes* serotype 1/2c (LM 0048-0305). Analyses of banding patterns were done with the GelCompar® II version 6.5 (BioNumerics, Applied-Maths). Similarity clustering analyses were performed with BioNumerics by using the unweighted pair group-matching aligorithm (UPGMA) and the Dice correlation coefficient with a tolerance of 1% (Chou and Wang 2006; Gianfranceschi *et al*. 2009).



*Salmonella braenderup* strain H9812 is used as the global standard reference in the PulseNet protocol (Gianfranceschi *et al*. 2009) and this strain was acquired from the Institute of Environmental Science and Research's reference culture collection in New

Zealand. DNA plugs were prepared in the same way for this strain as described in this study. Initially, a banding pattern was obtained with this strain when digested with *Xba*I (Roche Diagnostics) but in subsequent experiments, shearing was observed. Multiple attempts were made to optimize conditions for this strain, including altering switch times, preparing fresh chemicals/reagents for PFGE, fresh enzyme and buffer, preparing new plugs with fresh cultures and adding thiourea to the buffer, which is suggested for PFGE should shearing be the outcome as a result of strains that appear non-typeable (Zhang *et al*. 2004b). Aside from the many advantages to using this strain as a marker, there are disadvantages, some of which include that the protocol for PFGE preparation for *S. braenderup* is different to that of *Listeria* and some other organisms, a different enzyme namely *Xba*I is required for digestion of this strain and electrophoresis conditions for this strain may also be different to the organism of interest being examined (Hunter *et al*. 2005).



Careful consideration was given to each step in the protocol yet all attempts failed to produce positive PFGE results for *S. braenderup*. Since PFGE results were positive for *L. monocytgenes* but negative for *S. braenderup* strains, it was decided to normalize gels with *L. monocytogenes* serotype 1/2c (LM 0048-0305). Since the costs involved in preparing one gel for PFGE is large and the procedure is very time consuming, no more time and resources could be spent on optimizing conditions for *S. braenderup*.

Failure to successfully incorporate *S. braenderup* as the reference marker for PFGE was experienced by researchers from other local research institutions as well. They observed the same pattern of shearing.

## 7.4 RESULTS AND DISCUSSION

The enzyme *Apa*I was the restriction enzyme selected to cut the genomic DNA. It was demonstrated that *Apa*I and *Asc*I provides excellent discrimination for *L. monocytogenes* (Graves and Swaminathan 2001; Chou and Wang 2006). A comparison of the two enzymes revealed that a poorer discriminatory power was produced with *Asc*I (Chou and Wang 2006). The PulseNet protocol was modified slightly whereby a FastDigest *Apa*I was used which cut the DNA within 20 min as opposed to the 2h recommended by the protocol. Since PFGE protocols are long, improvements in the protocols are needed for a faster turnaround time for epidemiological surveillance (Graves and Swaminathan 2001).



Prior to the acquisition of the CHEF-DR II (Bio-Rad laboratories) system, the TAFE unit (OMEG Scientific) was available for PFGE analysis. This system required extensive optimization since the PulseNet protocol was not adapted for this system. After numerous attempts with altering switch times and voltage, a final switch time of 15 to 18 s was applicable for PFGE with a voltage of 120V and a run time of 18 h. This system had many ‘man-made’ components to substitute for parts missing and this could explain why the results were not reproducible. Initially, when DNA agarose plugs were prepared for PFGE with the TAFE unit, a banding profile was obtained when these DNA plugs underwent electrophoresis. Subsequent testing with the TAFE unit using the same DNA plugs produced negative results (shearing). The experiment was repeated numerous times, taking into account the many variables that could account for negative results,

including switch times, chemical reagents and the ‘quality’ of the enzyme. These were all changed but results still remained negative and the positive results were never again reproduced which led to the conclusion that the unit itself was faulty. The CHEF-DR II (Bio-Rad laboratories) apparatus became available for use and all the DNA agarose plugs that underwent electrophoresis in the TAFE unit was subjected to electrophoresis in the CHEF-DR II apparatus. Positive results were now obtained with the CHEF-DR II apparatus which indicated that the TAFE unit was indeed faulty.

As previously mentioned, 110 (98%) of guacamole isolates were characterized as lineage type II, whereas two (2%) were characterized as lineage type I. From the 98% lineage type II isolates, not all were selected for PFGE analysis. To save on costs and resources, but mainly to get an idea of the genetic diversity within the guacamole food processing plant, 47 isolates were selected (including the two from lineage I). The aim was to examine the genetic diversity of *L. monocytogenes* within the guacamole processing plant and then compare it to the genetic diversity of isolates within the other food processing plants. Guacamole isolates were obtained from a food processing plant in the Limpopo Province. The acquisition of the guacamole samples from Limpopo has expanded the geographical representation among the isolates in this study. All the isolates obtained from humans and other food and environmental sources were included (as specified in chapter 5). The *L. monocytogenes* isolate designation is also listed in Table 7.1.

Cluster analysis of the isolates as well as lineage group classification is depicted in the dendogram (Figure 7.2) which was constructed by the UPGMA with a tolerance of 1% in the band position applied (Gianfranceschi *et al*. 2009). Strains with the same PFGE type (fingerprint/pulsotype) were regarded as undistinguishable (zero band differences) (Zhang *et al*. 2004b). Twenty-four pulsotypes were identified among the 102 strains analyzed.

All the guacamole isolates were from one food processing plant and of the 47 isolates, 33 had the same pulsotype (this was the pulsotype that predominated among the guacamole isolates tested and included one water sample that was used to rinse the avocados). Although it was the most common pulsotype among the guacamole isolates selected, it did not occur in isolates from other sources. Interestingly, a sample used to rinse the avocado with the designation 'water accumulation point (avocado rinse)' had this same common pulsotype. This could be a clue to the origin of the guacamole contamination. The actual fruit, the avocado could harbour the *L. monocytogenes* on its skin and through a process of cross-contamination from the skin to the flesh, the finished product, the guacamole could become contaminated (Gregerson 2009). A further 12 guacamole isolates had another pulsotype and the two isolates from lineage I had the same pulsotype. In total, from the guacamole food and environmental processing environment, 4 different pulsotypes were identified and an overall level of similarity of greater than 86.8% of the lineage II guacamole isolates was revealed. It is important to determine whether a strain was introduced or whether one strain may persist in the food processing

environment (Miettinen *et al*. 1999). As a result effective sanitation efforts directed to the site of contamination can take place in order to eradicate the pathogen from the environment. The guacamole isolates are genetically similar with the exception of the two isolates from lineage I origin. Assuming that the skin of the avocados are the vehicle of contamination, many different *L. monocytogenes* strains may be introduced to the food processing environment and if adequate control measures are not in place, different strains may persist in the food processing environment. These strains may produce genetic variants, which arise from one common ancestor (Wiedmann 2002; Graves *et al*. 2005).



The food isolate obtained from an outside laboratory (food 25) was most closely related to the isolates from the guacamole food processing plant. Seven pulsotypes were identified from the meat isolates, one of which was characterized as a lineage I isolate by PCR-RFLP analysis. A comparison of the PFGE patterns of the meat isolates within lineage II indicated a broad genetic diversity within this group of isolates. Not all meat isolates were grouped within one cluster, showing the genetic diversity of isolates adapted to one food type. The exact origins of the isolates from the meat samples were unknown.

Within lineage group I, the *L. monocytogenes* isolate from milk had the same pulsotype as the isolates from coleslaw, spring onion, legume salad, barley, the cabbage shredding machine and the soup filler. The milk isolate was from a different geographical area

indicating that these strains were not correlated (Gianfranceschi *et al*. 2007). Ruminant healthy carriers may shed *Listeria* in faeces and milk (Esteban *et al*. 2009) however post-processing contamination may also occur. The isolate from the snoek mince, which was acquired from the plant next to the salad and vegetable food proessing environment, was genetically most similar to the isolates from the Danish feta salad and the Greek wheatberry salad. These isolates were collected from the same geographic area (close proximity) and over the same time period. *L. monocytogenes* is present in the food processing environment of the salad and vegetable plant. The fact that the cabbage shredding machine, soup fillers and spatula for scooping coleslaw were equipment contaminated with *L. monocytogenes*, implies that any RTE salad and vegetable product coming into contact with these equipment will be contaminated. Persistent in-house contamination may occur if effective measures for the eradication of the pathogen are not implemented. One of the isolates from the salad and vegetable food processing environment; namely, WIP chiller water unit, was classified as a lineage I isolate by PCR-RFLP analysis. This is the only isolate that was clustered with the isolates from the lineage II group and was genetically more similar to isolates within this group (Figure 7.2).Otherwise dendogram analysis showed that PFGE yielded a good binary division into genetic lineage I and II (classified by PCR-RFLP analysis).



Within lineage I, four of the seven human clinical isolates had the same pulsotype (clinical isolates 2, 5, 6 and 7) but all seven isolates were very closely related. No food isolate shared the same pulsotype as the four clinical isolates with the same pulsotype.

Very interestingly, a comparison of the pulsotype of the *L. monocytogenes* isolate from rooibos tea leaves and the isolate from human listeriosis (clinical isolate 3) revealed that they matched. Clinical isolate 1 only differed from clinical isolate 3 by the absence of one band. Since isolates from human listeriosis cases are regarded as more virulent and pathogenic, perhaps more attention needs to be given to the sampling number and regular microbiological examination within this rooibos tea processing plant. This was the only food isolate that had the same pulsotype as a human isolate. However, a pulsotype from a food source that is identical to a human isolate does not imply that there was a link between the consumption of the food type and the illness. What needs to be considered in outbreak investigations are epidemiological data together with the mocelcular sub-typing information in order to determine the relationship between isolates (Wiedmann 2002). Similar or identical isolates can be enumerated from different food types from a different geographic source and time period, therefore identical patterns of isolates from foods and humans does not imply that a specific food type is the vehicle of infection. For that to be confirmed, further microbiological and epidemiological tests need to be performed (Gianfranceschi *et al*. 2009). Within lineage I, *L. monocytogenes* serotypes 1/2b and 4b are responsible for the majority of human listeriosis cases (Miettinen *et al*. 1999).



Pulsotypes differed in their association with different sources and geographical areas. It has been established that isolates from different sources form distinct yet overlapping populations (Gianfranceschi *et al*. 2009). Thirteen pulsotypes (e.g snoek mince, mixed trough, food 25) were represented only by a single isolate i.e the pulsotype occurred only

once. No one pulsotype was widely distributed between processing plants. Undistinguishable pulsotypes were mainly confined within a single processing plant possibly identifying in-plant persistent contamination. The exception was the milk isolate which had a pulsotype identical to isolates from the salad and vegetable food processing plant. Pulsotypes within one lineage group were closer related by cluster analysis grouping.

The *L. monocytogenes* strain (stored as a glycerol stock) from the chicken blood that was characterized as a lineage II isolate together with the two isolates from the trout terrine food samples characterized as lineage I by PCR-RFLP analysis were unfortunately lost and the attempt to revitalize them from the culture itself was unsuccessful. Unfortunately, a PFGE fingerprint type is not available for these isolates.

**Table 7.1** A list of the *L. monocytogenes* isolates that underwent PFGE analysis

| ***L. monocytogenes* isolate designation** |
|---|
| Clinical isolates 1-7 |
| Guacamole isolates 7, 8, 9, 10, 11, 12, 13, 15, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28, 29, 30, 31, 35, 36, 37, 38, 39, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 66, 80, 89, 209 (3), 214 (1), 216 (1), 219 (1), 223, 233, 245 |
| Carrot from coleslaw |
| Carrot |
| Coleslaw (x3) |
| Danish feta salad |
| Spring onion after dipping (peroxyacetic acid 150 ppm) |
| Spring onion |
| Spelt and legume salad – made with 'stampkoring' |
| Hulled barley salad (made with pearl barley) |
| Greek wheatberry salad (made with chickpeas) |
| Legume salad |
| Barley salad |
| Snoek mince |
| Meat (17 samples) |
| Milk (underwent UV treatment) |
| Left soup filler (machine used to fill bags with soup) |
| Soup filler |
| Cabbage shredding machine |
| Kitchen drain (x3) (salad and vegetable food processing environment) |
| Spatula for scooping coleslaw (x2) |
| Mixed trough – trough used for mixing coleslaw in (x2) |
| A swab sample from work in process (WIP) chiller water unit – a holding chiller for vegetables |
| WIP chiller water unit |
| Water accumulation points (avocado rinse) |
| Water accumulation points (Drain 2a) (guacamole processing plant) |
| Food 25 (unknown food type from outside laboratory) |
| Rooibos tea leaves |

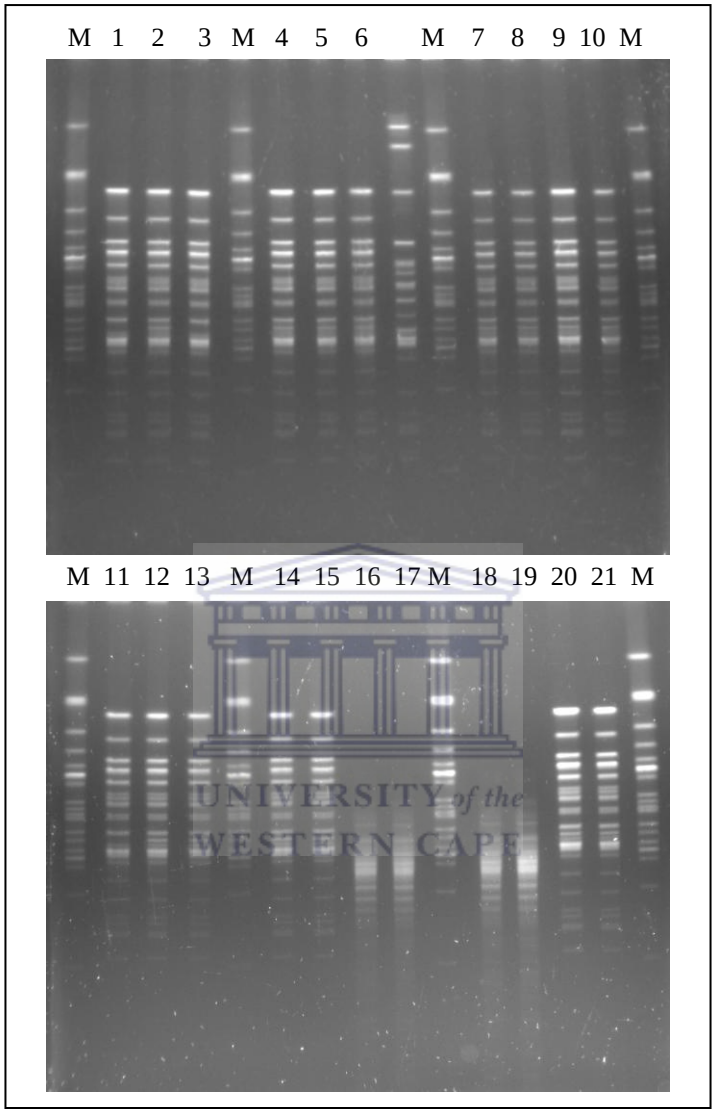





**Figure 7.1a** PFGE with enzyme *Apa*I of guacamole isolates. **M**: reference strain serotype 1/2c (CIP 105.448); **Lane 1**: guacamole isolate 9*; **lane 2**: guacamole isolate 10; **lane 3**: guacamole isolate 11; **lane 4**: guacamole isolate 12; **lane 5**: guacamole isolate 13; **lane 6**: guacamole isolate 15; **lane 7**: guacamole isolate 18; **lane 8**: guacamole isolate 19; **lane 9**: guacamole isolate 20; **lane 10**: guacamole isolate 21, **lane 11**: guacamole isolate 22; **lane 12**: guacamole isolate 23; **lane 13**: guacamole isolate 25; **lane 14**: guacamole isolate 26; **lane 15**: guacamole isolate 27; **lane 16-19**: new DNA plugs were prepared for these strains 28, 29, 30, 31 and the PFGE was redone; **lane 20**: guacamole isolate 35; **lane 21**: guacamole isolate 36. *guacamole isolate 8 had the same pulsotype as that represented in all the other strains above (data not included).

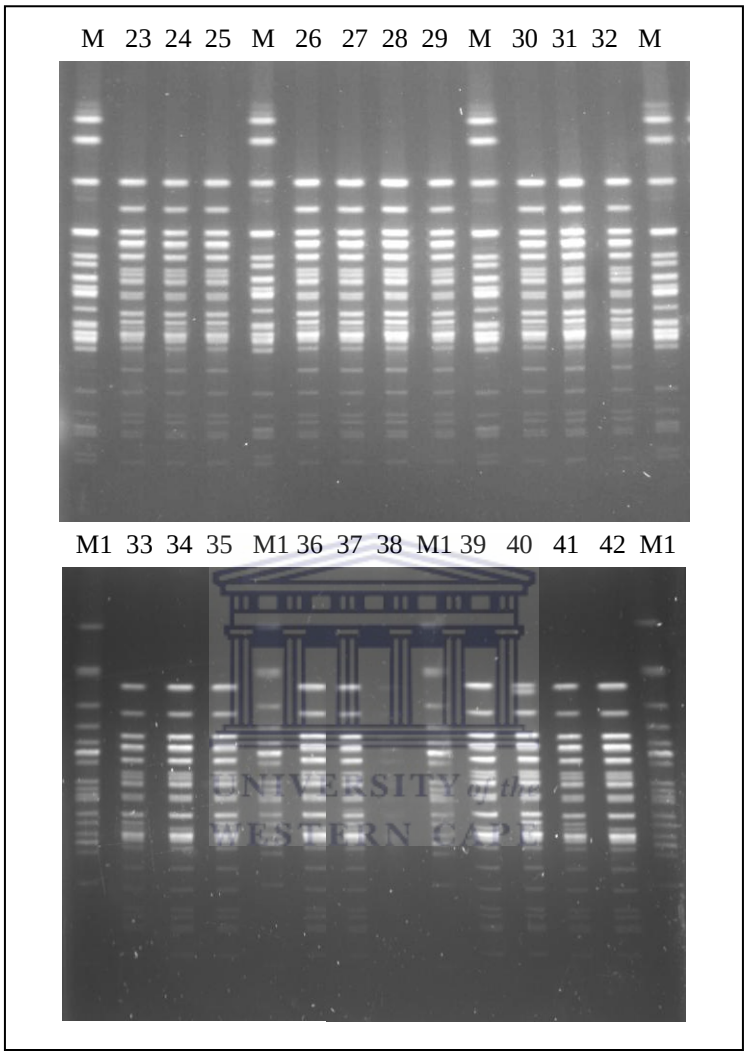



**Figure 7.1b** PFGE with enzyme *Apa*I of guacamole isolates. **M**: reference strain serotype 1/2c (LM 0048-0305); **Lane 23**: guacamole isolate 28; **lane 24**: guacamole isolate 29; **lane 25**: guacamole isolate 30; **lane 26**: guacamole isolate 31; **lane 27**: guacamole isolate 66; **lane 28**: guacamole isolate 80; **lane 29**: guacamole isolate 88; **lane 30**: guacamole isolate 214; **lane 31**: guacamole isolate 216; **lane 32**: guacamole isolate 219; **M1**: reference strain serotype 1/2c (CIP 105.448); **lane 33**: guacamole isolate 37; **lane 34**: guacamole isolate 38; **lane 35**: guacamole isolate 39; **lane 36**: guacamole isolate 40; **lane 37**: guacamole isolate 42; **lane 38**: guacamole isolate 43; **lane 39**: guacamole isolate 44; **lane 40**: guacamole isolate 45; **lane 41**: guacamole isolate 46; **lane 42**: guacamole isolate 47

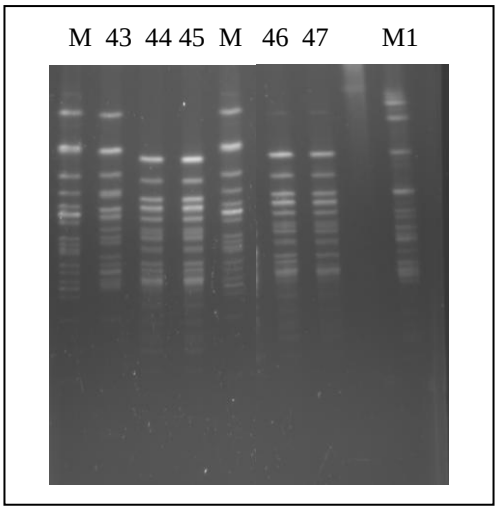



**Figure 7.1c** PFGE with enzyme *Apa*I of guacamole isolates. **M**: reference strain serotype 1/2c (CIP 105.448); **M1**: reference strain serotype 1/2c (LM 0048-0305); **lane 43**: guacamole isolate 50*; **lane 44**: guacamole isolate 51; **lane 45**: guacamole isolate 209; **lane 46**: guacamole isolate 223; **lane 47**: guacamole isolate 233

* guacamole isolate 7 had the same pulsotype as displayed by isolate 50 (data not shown)



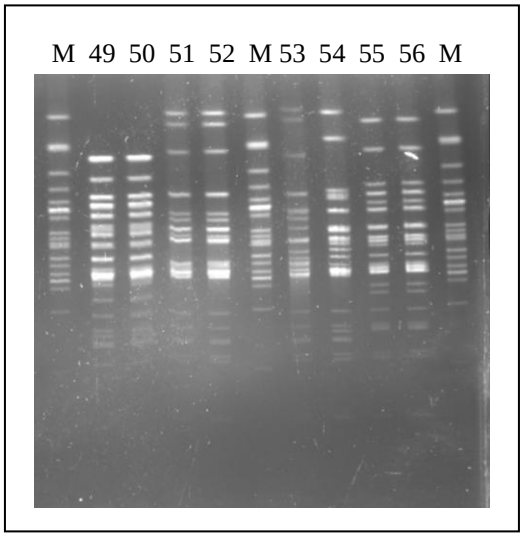



**Figure 7.1d** PFGE with enzyme *Apa*I of guacamole isolates and meat isolates. **M**: reference strain serotype 1/2c (CIP 105.448); **M**: reference strain serotype 1/2c (LM 0048-0305); **lane 49**: guacamole isolate 48; **lane 50**: guacamole isolate 49; **lanes 51-56**: meat isolates

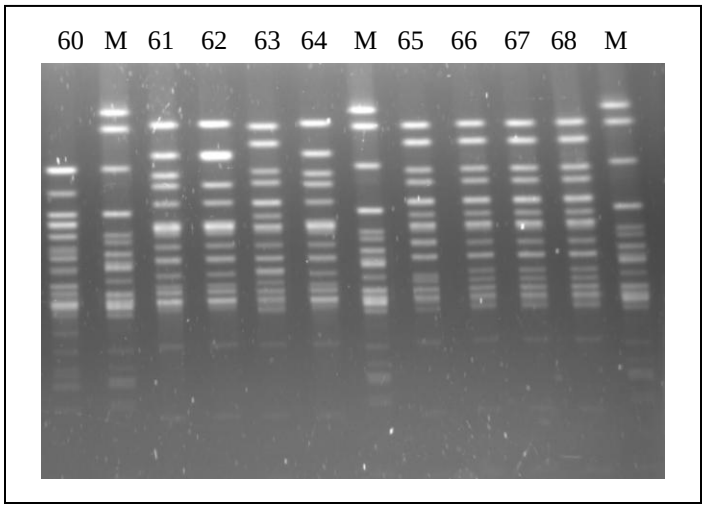



**Figure 7.1e** PFGE with enzyme *Apa*I of guacamole isolate, rooibos tea isolate clinical isolates. **M**: reference strain serotype 1/2c (LM 0048-0305); **lane 60**: guacamole isolate 245; **lane 61**: rooibos tea isolate; **lane 62**: clinical isolate 1; **lane 63**: clinical isolate 2; **lane 64**: clinical isolate 3; **lane 65**: clinical isolate 4; **lane 66**: clinical isolate 5; **lane 67**: clinical isolate 6; **lane 68**: clinical isolate 7



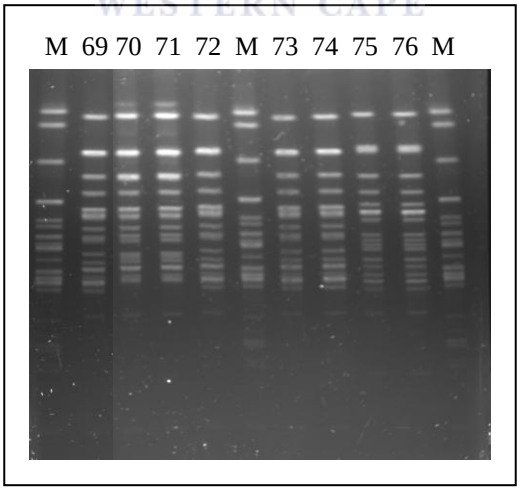



**Figure 7.1f** PFGE with enzyme *Apa*I of fruit and vegetable isolates. **M**: reference strain serotype 1/2c (LM 0048-0305); **lane 69**: coleslaw isolate; **lane 70**: carrot from coleslaw isolate; **lane 71**: carrot isolate; **lane 72**: spring onion after dipping isolate; **lane 73**: spring onion isolate; **lane 74**: legume salad isolate; **lane 75**: Danish feta salad isolate; **lane 76**: Greek wheatberry salad isolate

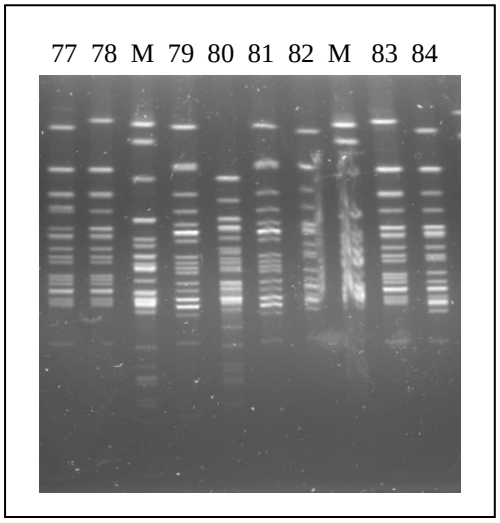



**Figure 7.1g** PFGE with enzyme *Apa*I of isolates from the fruit and vegetable processing environment. **M**: reference strain serotype 1/2c (LM 0048-0305); **lane 77**: isolate from kitchen drain; **lane 78**: isolate from kitchen drain; **lane 79**: isolate from spatula; **lane 80**: non-*L. monocytogenes* isolate (culture regrown and rerun on another gel); **lane 81**: isolate from spatula; **lane 82**: isolate from soup filler; **lane 83**: isolate from kitchen drain; **lane 84**: isolate from cabbage shredding machine



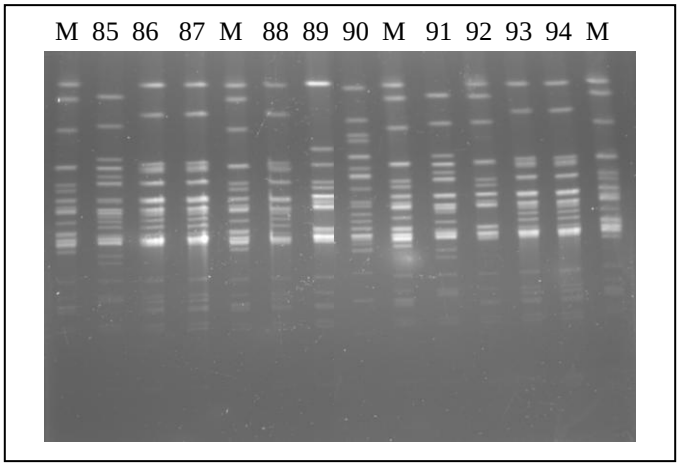



**Figure 7.1h** PFGE with enzyme *Apa*I of isolates from meat. **M**: reference strain serotype 1/2c (LM 0048-0305); **lanes 85-94**: meat isolates; **lane 90**: only meat isolate characterized as lineage type

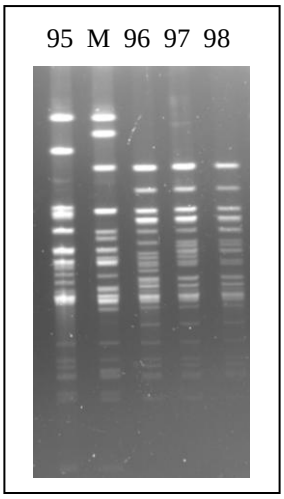



**Figure 7.1i** PFGE with enzyme *Apa*I of isolates from various environments. **M**: reference strain serotype 1/2c (LM 0048-0305); **lane 95**: meat isolate; **lane 96**: isolate food 25 (unknown food isolate from outside laboratoty); **lane 97**: isolate from water accumulation point of avocado rinse (drain 2a); **lane 98**: water accumulation point (avocado rinse)



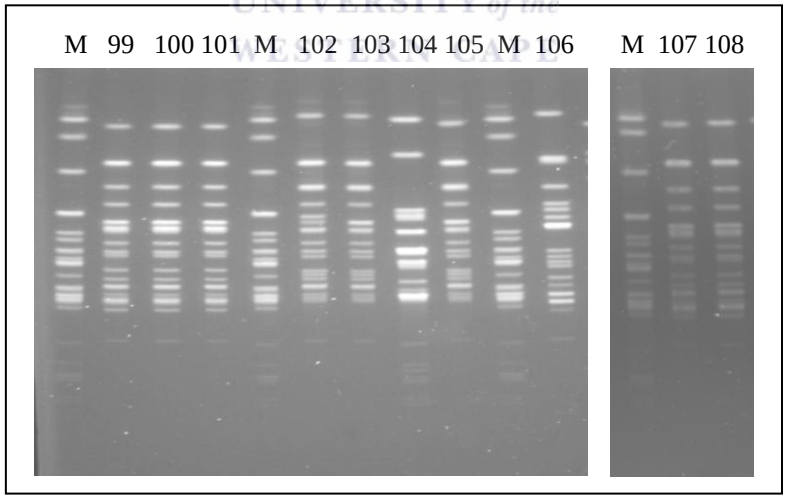



**Figure 7.1j** PFGE with enzyme *Apa*I of isolates from various environments. **M**: reference strain serotype 1/2c (LM 0048-0305); **lane 99**: coleslaw isolate; **lane 100**: coleslaw isolate; **lane 101**: left soup filler isolate; **lane 102**: isolate from mixed trough (used to mix coleslaw in); **lane 103**: isolate from spelt and legume; **lane 104**: isolate from WIP chiller (a holding chiller for vegetables); **lane 105**: isolate from WIP chiller; **lane 106**: isolate from mixed trough; **lane 107**: isolate from barley salad; **lane 108**: isolate from hulled barley salad

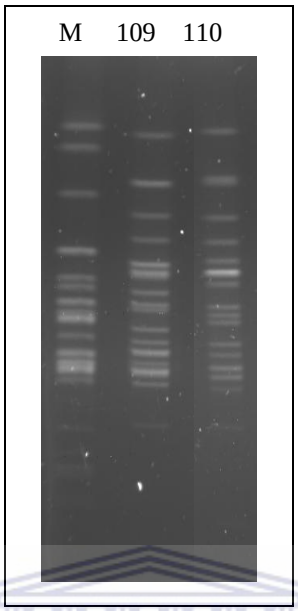



**Figure 7.1k** PFGE of isolates from milk and snoek mince with enzyme *Apa*I. **M**: reference strain serotype 1/2c (LM 0048-0305); **lane 109**: milk isolate; **lane 110**: snoek mince isolate

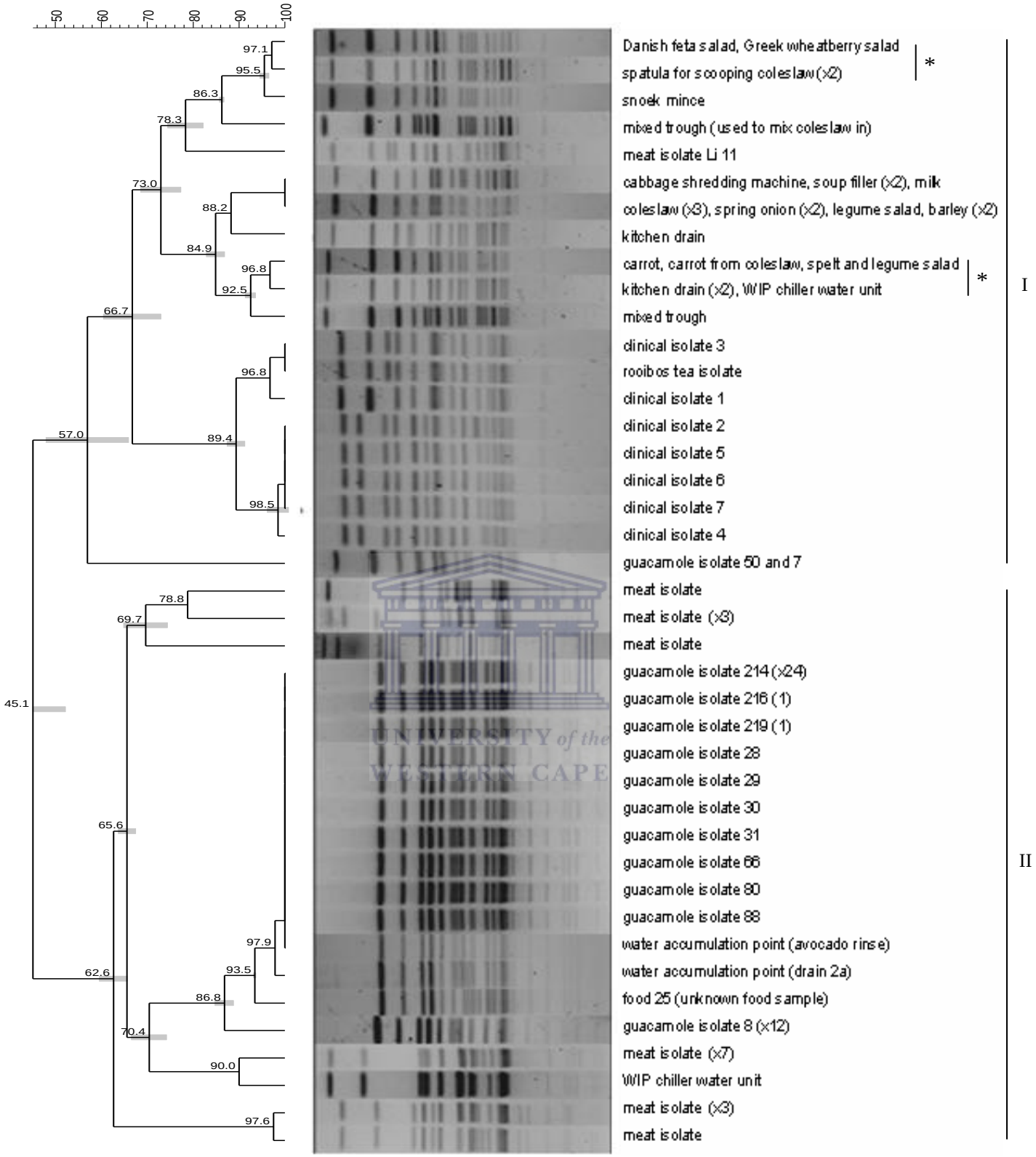



**Figure 7.2** PFGE based dendogram (UPGMA cluster analysis based on dice correlation coefficient) demonstrating the genetic diversity of various *Apa*I digested *L. monocytogenes* isolates.

* These isolates had the same pulsotype respectively, although cluster analysis did not indicate 100% similarity

Despite its relevance, the drawback of PFGE is that it requires pure cultures of *L. monocytogenes* and the technique is also time-consuming (Schmid *et al*. 2003) and labour intensive (Doumith *et al*. 2004). It also cannot be modified to target specific polymorphisms of interest which makes alternative, additional DNA sub-typing methods beneficial for sub-typing food-borne pathogens (Ducey *et al*. 2007). In order to minimize the time spent on PFGE, FastDiget enzyme *Apa*I was used for restriction digestion of the DNA plugs. The PulseNet protocol was modified to reduce restriction digestion time from 2 h to only 20 min.



Although the *L. monocytogenes* reference strains were included for PFGE analysis, it was not possible to assign serotype to the isolates from the different food processing environments based on the PFGE banding patterns of the reference isolates. Serotype can be predicted but this requires very large databases of data from strains that have been serotyped by other means. Even then, PFGE diversity is such that many isolates will have novel patterns and as a result it is not possible to predict serotype. For example, *L. monocytogenes* serotype 1/2c (CIP 105.448 and LM 0048-0305) from two different geographic locations had different PFGE pulsotypes (data not shown).

Generally, PFGE fingerprints that were identical belonged to the same serotype (Chou and Wang 2006). In a study conducted by Revazishvili *et al*. (2004), it was determined that most of the strains with identical pulsotypes belonged to a single serotype; however, some strains that were genetically identical or very closely related with the same

pulsotype belonged to different serotypes. This suggested that the strains diverged from the same ancestor into different serotypes by a process of antigen switching, a mechanism displayed by other bacterial pathogens. This implies that the assumption about the pathogenic potential and genetic relatedness of strains based solely on their serotype designation should be reconsidered especially regarding the strains considered to have reduced virulence or avirulence (i.e. non- 1/2a, 1/2b and 4b).

Due to the very large distribution of *L. monocytogenes* strains involved in human listeriosis cases and in various environments, PFGE is required and very useful in developing a better understanding of the diversiy of strains adapted to these environments as well as determining listeriosis outbreak sources (Fugett *et al.* 2007).

## 7.5 CONCLUSION

Where contamination in a food plant occurs, it is important to determine whether the contamination was introduced or whether one strain persists in the food processing environment. This information is important in order to implement effective cleaning and sanitation efforts at the areas of contamination so that *L. monocytogenes* can be eradicated from the food processing plant. Strains can become resident in the food processing plant for a long enough time to produce genetic variants. They may form biofilms and persist in the environment for long periods.



Pulsotypes from human, food and environmental *L. monocytogenes* isolates from different geographical locations were compared in order to assess whether certain strains were associated with certain foods and to determine their similarity or association to the isolates from human cases. Information pertaining to geographical or temporal association of isolates could also be obtained. The PFGE profiles of 102 *L. monocytogenes* isolates were determined. The isolates were divided into 24 *Apa*I pulsotypes (level of relatedness 45.1-100%). Isolates were considered to have the same pulsotype when the number and positions of the bands were undistinguishable.

*Listeria monocytogenes* contamination within the salad and vegetable food processing plant was the result of lineage I isolates. Cluster analysis indicated that the strains were genetically similar with a level of relatedness of up to 100% observed. Cross

contamination seems very likely in this plant as the equipment and machines used for food preparation were all contaminated and some shared the same pulsotype as present in some food types. The isolates enumerated from the kitchen drain as well as the chiller water unit (a holding chiller unit for vegetables) had the same pulsotype as the carrot, carrot from coleslaw and spelt and legume salad food types. The spatula for scooping coleslaw had the same pulsotype as the Danish feta salad and Greek wheatberry salad food types. In these cases, the food may contaminate the equipment and machinery which in turn contaminates every other food type exposed to the same equipment and machinery. Effective de-contamination procedures may need to be applied in this instance as contamination may be persistent in-house contamination. Bioflms can persist in food processing plants for up to more than ten years (Fugett *et al*. 2007).



All the guacamole isolates were from an area in the Limpopo Province and of the 47 isolates, 45 were a representation of lineage II isolates previously determined by PCR-RFLP analysis. Of the total, 33 isolates had the same pulsotype (this was the pulsotype that predominated among the guacamole isolates tested and included one water sample that was used to rinse the avocados). The remaining 12 had one common pulsotype and two were determined as lineage I isolates.

All the clinical isolates were characterized as lineage I isolates. Compared to lineage II isolates, lineage I isolates are said to be overrepresented among human clinical cases. Lineage II isolates are mainly implicated in foods (Fugett *et al*. 2007). One food isolate

(from rooibos tea leaves) had the same pulsotype as an isolate from a human clinical case. However, it cannot be concluded that this food type is a vehicle of contamination and linked to the clinical case as many food isolates may share fingerprints that are identical. Consideration also has to be given to the fact that boiling water is added to rooibos tea leaves in the preparation of the tea. The temperature of the water would be sufficient to kill the pathogen present and render the product safe for human consumption. On the other hand, the isolate enumerated from the rooibos tea leaves could in fact be a cause for concern and further microbiological examination of the processing plant needs to be administered. This information is very useful in helping to detect potential outbreak sources. It is important to develop a better understanding of the *L. monocytogenes* PFGE type diversity from various sources since there are many disease associated strains.



Large PFGE databases representing isolates from various sources around the world would assist in the interpretation of subtype data for epidemiological purposes and would aid in determining the prevalence of the strain in the environment; whether it is widespread or specific to the source from where it was isolated. PFGE databases would have more value the more isolates and fingerprints are added from environmental sources in order to better understand the diversity of *L. monocytogenes* as a food-borne pathogen.

PFGE data are useful in outbreak investigations and to determine areas of cross-contamination. Information regarding in-house *L. monocytogenes* contamination is

helpful to prevent finished product contamination. This information would assist in detecting and eliminating the outbreak source (Wiedmann 2002). As previously mentioned; PFGE subtype matches between a food or environmental isolate with a human isolate, alone does not imply a causal relationship. However, the more common a pulsotype linked to an outbreak is, the stronger the evidence is for linking the outbreak to the food source. Additional sub-typing methods are beneficial for further characterization of isolates. PFGE databases may need to contain information on thousands of isolates from various and broad geographic regions in order to interpret subtype data.

This study has formed the basis for generating information about the distribution, diversity and ecology of *L. monocytogenes* strains in food, the environment and human clinical cases and will also pave the way for more research to be undertaken in the future, especially in Sub-Saharan Africa.

# CHAPTER 8

## 8.1 CONCLUSION

*Listeria monocytogenes* is a pathogen that has caused an increase for concern in the food processing environment of late. The ability of the organism to form biofilms in the food processing environment enhanced its ability to withstand or survive cleaning or disinfection procedures. The presence of *Listeria* spp. in the food processing environment may be an indicator for very low numbers of *L. monocytogenes* that may be overlooked since this pathogenic strain is usually outgrown by the other non-pathogenic *Listeria* strains. This is reason for concern since very low numbers of *L. monocytogenes* can grow during storage of foods, especially at refrigeration temperatures at which *Listeria* is able to survive and grow. It is then important that many areas along the production line in a food processing plant be tested for *Listeria* including condensation on the floor and areas beneath machinery since the organism can so easily enter from the outside environment.

Control measures for the presence of *L. monocytogenes* in foods are influenced by worldwide government policies. These policies differ since there are discrepancies regarding the number of cells that must be ingested for listeriosis to occur. Low tolerance or zero tolerance rulings for the presence of *L. monocytogenes* in foods that do not support the growth of this organism is debatable and differs between countries. For foods that do support the growth of *L. monocytogenes*, adequate control measures, including

HACCP, should be implemented to prevent the proliferation of this organism in the food product. Regulation policies, together with effective cleaning and sanitization practices are vital in order to ensure food safety.

It has been determined that some strains of *L. monocytogenes* are more virulent than others with serotypes 1/2a, 1/2b and 4b responsible for the majority of outbreaks of food-borne listeriosis worldwide. However, it has been reported that there are no surveillance reports or epidemiological data for these findings in Africa. *L. monocytogenes* isolates can be grouped relative to their outbreak potential. The serotypes of *L. monocytogenes* are known to be genetically diverse with differences in virulence potential being displayed by serotypes; however detailed knowledge of the diversity and evolution of isolates is still deficient. The aim of this research has focused on the application of both traditional and molecular based technology for epidemiologic investigation. These techniques, which possess enormous diagnostic potential, have been applied so that the result will be an increase in the knowledge of the distribution and diversity of *L. monocytogenes* strains in the food processing environment and food supply in and around the Western Cape and their implication in listeriosis outbreaks.



Molecular based methods have allowed for the rapid identification of *L. monocytogenes* in culture or directly in specimens. However, the need for traditional microbiological methods should not be underestimated or overlooked where viable isolates are required for sub-typing methods used for outbreak investigations. Complementing conventional

with molecular methods for epidemiology studies regarding food-borne outbreaks or in surveillance systems is fundamental for the prevention and control of human listeriosis.

Multiple typing methods are generally used to subtype *L. monocytogenes* isolates to confirm its identity. Serotyping has always been the typing method of choice, however the results obtained with serotyping may be discrepant since it is dependent on the phenotypic characteristics of the bacteria and as a result, alternative molecular techniques, which are more specific and sensitive, are utilized for the identification and differentiation between *L. monocytogenes* serotypes. Furthermore, serotyping is not only labor intensive but reagents are expensive and high quality antisera are required.



A PCR-RFLP technique was designed in order to differentiate between *L. monocytogenes* serotypes based on the presence of SNPs in the gene. Upon discovering that there are variations of SNPs within strains of the same serotypic designation in the *plcA* and *plcB* gene, the virulent *hly* gene was selected for further analysis. Sequencing of numerous isolates with the same serotypic designation showed that there were SNPs conserved between these strains but also shared with other *L. monocytogenes* serotypes. The *hly* gene showed to have a distinct, well conserved region with some variation in the central domain for the differentiation between 3 lineage groups The PCR-RFLP technique applied to the *hly* gene characterized *L. monocytogenes* isolates into three lineage groups. PCR-RFLPs with enzyme *Nde*I characterized *L. monocytogenes* serotypes 1/2b, 3b, 4b, 4d, 4e and 7 as lineage type I, enzyme *Hae*II characterized *L. monocytogenes* serotypes

1/2a, 1/2c, 3a and 3c as lineage type II and enzyme *Bsh*1285I characterized *L. monocytogenes* serotypes 4a and 4c as lineage type III. This PCR-RFLP molecular technique provided a sensitive, discriminatory and reproducible method for epidemiological investigations of *L. monocytogenes* based on lineage group distinction of *L. monocytogenes* isolates. MAMA-PCR was another molecular method applied for lineage group distinction of isolates. This method confirmed that the PCR-RFLP technique applied in this study for lineage group distinction of *L. monocytogenes* serotypes was conclusive.

A total of 172 *L. monocytogenes* positive samples were included in this study. They included isolates from human clinical cases (n = 7), guacamole (n = 112), salad and vegetables (n = 13), seafood (n = 3), environmental (n = 16), meat (n = 17), rooibos tea (n = 1), milk (n = 1), chicken blood (n = 1) and Food 25 (n = 1, unknown food sample from an outside laboratory). All samples tested positive for the *hly* gene by PCR amplification. PCR-RFLPs characterized the seven human clinical isolates as lineage type I which is in agreement with literature that show lineage I isolates to predominantly be responsible for human listeriosis, indicating a higher virulence potential in these strains. From the 165 *L. monocytogenes* isolates from food and environmental sources, 148 samples were representative of food isolates and from this total, 14% were characterized as lineage I and 86% as lineage II. Isolates from lineage II could be suggestive of a persistent environment contamination. Various molecular sub-typing data have shown that *L. monocytogenes* can persist in the food processing environments for very long periods of

time (beyond 10 years). These results comply with research which indicates that lineage II isolates show an over-representation among food and environmental samples and fewer in human disease.

Several other studies show that most food isolates are characterized as lineage II strains and strains of this lineage appear less likely to cause human disease compared to lineage I strains. However, it is unlikely that a clear division between food and clinical isolates can be established since listeriosis in humans is mainly acquired through contaminated foods. As mentioned previously, other research has shown that *InlA* with premature stop codons are represented among much food isolates which supports why many food isolates show reduced virulence. Furthermore, since isolates from food and human cases may undergo mutations in their genetic material due to different environmental pressures they may differ to some extent in their serotypes. As a result, the *L. monocytogenes* serotype from food responsible for the human illness may have a different serotype to the isolate from the human listeriosis case. This implies that the assumption about the pathogenic potential and genetic relatedness of strains based solely on their serotype designation should be reconsidered especially regarding the strains considered to have reduced virulence or avirulence (i.e. non- 1/2a, 1/2b and 4b).



PFGE is currently the method of choice for investigating food-borne outbreaks of listeriosis and for determining the source of contamination and the outbreak strain (Revazishvili *et al*. 2004). The discriminatory power of this technique is critical for

human disease outbreak investigations. The large number of PFGE types found among *L. monocytogenes* isolates indicates the need for the acquisition of information of numerous isolates from various geographic sources to include in the PFGE database in order to give perspective to epidemiological investigations. PFGE is useful in determining which subtypes were persistent in the food processing environment and causing the contamination. These fingerprint databases may be very useful in tracing vehicles of infection by comparing human isolates with food isolates. The *L. monocytogenes* isolates from this study were divided into 24 *Apa*I pulsotypes. The seven clinical isolates (of lineage I origin), indicate that isolates from this lineage group contribute to the real burden of disease. The few human listeriosis isolates obtained from Groote Schuur Hospital over the three year period points to a low degree of listeriosis outbreaks or otherwise cases that are not reported in the Western Cape. Four of the seven human isolates had the same pulsotype. Generally, most strains with identical pulsotypes belong to a single serotype (Chou and Wang 2006). Since four of the seven human isolates had identical lineage I PFGE fingerprints, one could assume that the same strain was responsible for the illness; however there may be exceptions. The *L. monocytogenes* isolate from the rooibos tea was the only food isolate that had a pulsotype identical to the human strain; both isolates were of lineage I origin. Within this lineage, isolates either have a 1/2b, 3b, 4b, 4d or 4e serotypic designation. However, as previously stated, within this lineage it's predominantly 1/2b and 4b that is associated with food-borne listeriosis with serotype 4b responsible for the major human epidemic cases. The PFGE cluster analysis results from this study show that within lineage I, all clinical isolates had a

89.4% level of relatedness. A comparison of these pulsotypes with isolates linked to human listeriosis worldwide (in large PFGE databases) could determine whether these strains are also epidemic clones or whether they correspond to widely distributed clinical cases.

Isolates from different geographic locations were included in this study. Even where no evidence of the vehicle of infection is available, it is important that the public, especially high-risk individuals be advised on what food types to avoid. Dietary guidelines could be provided to these individuals, including pregnant women. For instance, numerous guacamole samples were contaminated with *L. monocytogenes* and this food type, together with many other food types analyzed in this study (including the salad and vegetables), are RTE food products that require no heat process prior to consumption. This implies that there is a risk associated with the consumption of these food types, especially for high-risk individuals. The PFGE results of isolates from food and the food processing environment show that some *L. monocytogenes* pulsotypes are associated with specific sources, indicative of possible in-house contamination. Of the total isolates analyzed by PFGE, 13 pulsotypes were represented only by a single isolate i.e. the pulsotype occurred only once. These single pulsotypes may; however be found in other sources if more isolates are characterized in the future. This highlights or stresses the need for large PFGE databases containing a large collection of food associated isolates. For example, these isolates that were represented by a singe pulsotype may even be the strains responsible for outbreaks or epidemics worldwide. It is important to develop a

better understanding of the *L. monocytogenes* PFGE type diversity from various sources since there are many disease associated strains. Alone, PFGE pulsotype matches between clinical and food/environmental sources do not necessarily imply a causal relationship; however, the more common a PFGE pulsotype from food is that is linked to a clinical isolate, the stronger the epidemiological evidence is to imply a causal relationship. Large PFGE databases that include patterns from sources around the world would help circumvent this problem.

Within the organic and battery chicken farming practices, a total of 56% chicken blood samples tested positive for the *hly* gene of *L. monocytogenes* by PCR amplification. As previously mentioned, the prevalence of different pathogens on South African poultry has not been researched well. The results of this study have demonstrated a high incidence of *L. monocytogenes* in chicken blood in the Western Cape region and the risk of infection associated with mishandling the raw poultry or improper/inadequate cooking. Also, viable *L. monocytogenes* was enumerated from the chicken blood which was recovered by culture based methods and identified as a lineage II isolate by PCR-RFLP analysis. These results show that it may be advisable to perform regular microbiological testing within the chicken farming industry as the presence of viable isolates within blood may be transferred to the flesh of the animal and cause food-borne illness should proper storage and cooking not be performed.

Practical applications for ensuring that *L. monocytogenes* listeriosis infection is minimized include; cooking food to an internal temperature of 70°C for more than 20 minutes to destroy the organism, reheating cooked food thoroughly (70°C), good personal hygiene which includes washing of hands and ensuring that food is stored correctly at fridge or freezer temperatures (Mahmood *et al*. 2003).

The methods used in this study highlighted the reliability and accuracy of both traditional and molecular tools such as species-specific PCR, PCR-RFLP analysis and PFGE to provide critical information about the presence of *L. monocytogenes* in food processing environments. The information collected can be used to take suitable steps in improving food safety as well as providing dietary advice to those who are at a greater risk for infection. Practical recommendations can be applied for the control of *L. monocytogenes* in the food processing environment. Furthermore, a standardized protocol for lineage group distinction of *L. monocytogenes* serotypes by PCR-RFLP analysis can be implemented. These findings have given an indication of the virulence potential of certain *L. monocytogenes* strains over others where molecular results showed lineage I isolates to be responsible for all cases of human listeriosis. The results generated have contributed to the worldwide data on the geographic distribution of *L. monocytogenes* lineage groups and strain diversity in food products - data that has not yet been generated in sub-Saharan Africa yet. The matching of one PFGE food pulsotype to a number of human listeriosis isolates provides important source tracking information for outbreak investigations. The findings could be used for outbreak investigations and as part of

surveillance efforts. In addition, information pertaining to the distribution, diversity and ecology of *L. monocytogenes* strains in food, the environment and human clinical cases obtained in this research will also pave the way and form the basis for more research to be undertaken in the future, especially in Sub-Saharan Africa.

# CHAPTER 9

## 9.1 REFERENCES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## Annexure 1

***Listeria monocytogenes*** **genome (Pubmed NCBI: accession number U25449) – primer binding sites identified in bold.**

gagaggaggg gctaaacagt atttggcatt attaggttaa aaaatgtaga aggagagtga
aacccatgaa aaaaataatg ctagttttta ttacacttat attagttagt ctaccaattg
cgcaacaaac tgaagcaaag gatgcatctg cattcaataa agaaaattta atttcatcca
tggcaccacc agcatctccg cctgcaagtc ctaagacgcc aatcgaaaag aaacacgcgg
atgaaatcga taagtatata caaggattgg attacaataa aaacaatgta ttagtatacc
acggagatgc agtgacaaat gtgccgccaa gaaaaggtta taaagatgga aatgaatata
tcgttgtgga gaaaaagaag aaatccatca atcaaaataa tgcagatatc caagttgtga
atgcaatttc gagcctaaca tatccaggtg ctctcgtgaa agcgaattcg gaattagtag
aaaatcaacc cgatgttctt cctgtcaaac gtgattcatt aacacttagc attgatttgc
caggaatgac taatcaagac aataaaattg ttgtaaaaaa tgctactaaa tcgaacgtta
acaacgcagt aaata**catta gtggaaagat ggaatg**aaaa atatgctcaa gcttatccaa
atgtaagtgc aaaaattgat tatgatgacg aaatggctta cagtgaatcg caattaattg
caaaatttgg tacggcattt aaagctgtaa ataatagctt gaatgtaaac ttcggcgcaa
tcagtgaagg gaaaatgcaa gaagaagtca ttagttttaa acaaatttac tataacgtga
atgttaatga acctacaaga ccttccagat ttttcggcaa agctgttact aaagagcagt
tgcaagcgct tggagtgaat gcagaaaatc ctcctgcata tatctcaagt gtggcatatg
gccgtcaagt ttatttgaaa ttatcaacta attcccatag tactaaagta aaagctgctt
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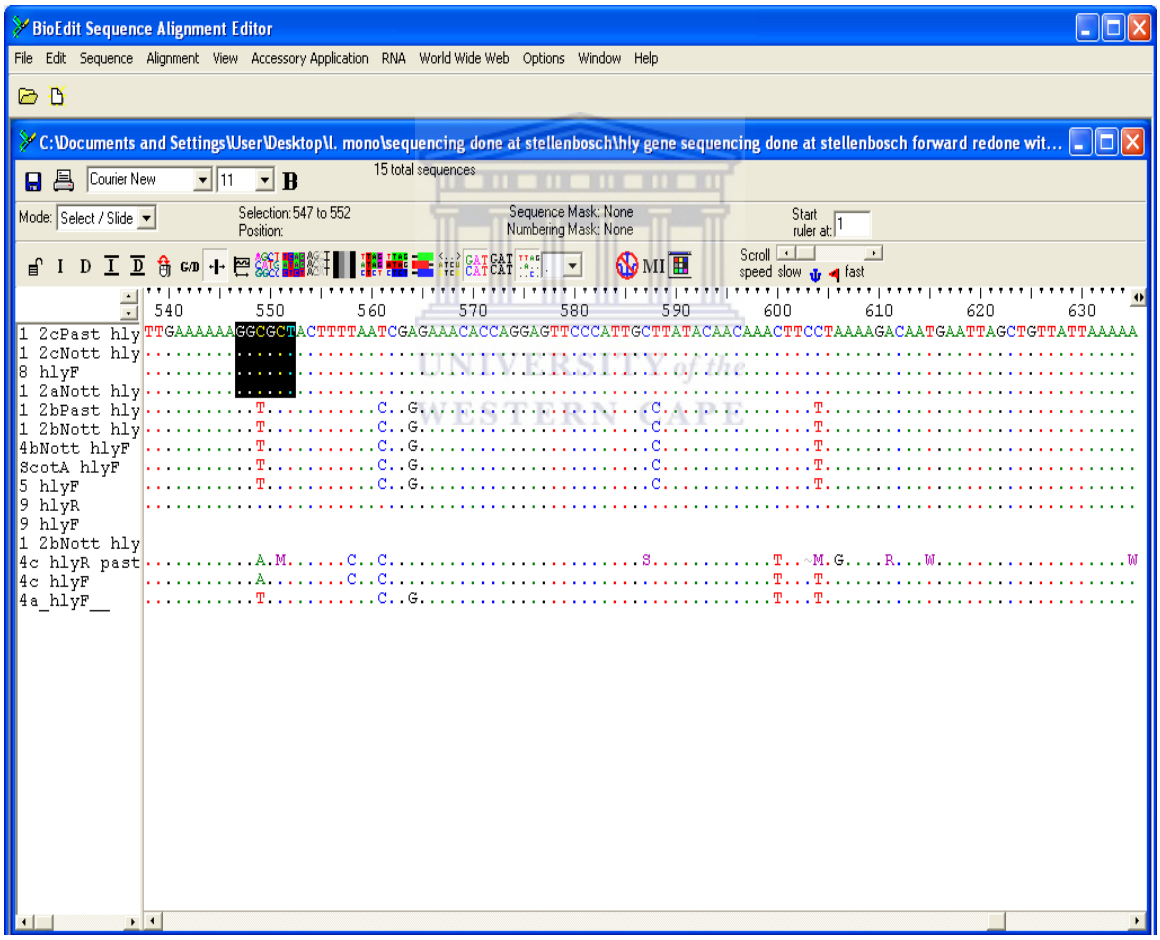
aaaattcttc cttcaaagcc gtaatttacg gtggctccgc aaaagatgaa gttcaaatca
tcgacggtaa cctcggagac ttacgagata ttttgaaaaa aggtgctact tttaaccggg
aaacaccagg agttcccatt gcctatacaa caaacttctt aaaagacaat gaattagctg
ttattaaaaa caactcagaa tatattgaaa caacttcaaa agcttataca gatggaaaaa
tcaaca**tcga tcactctgga ggatac**gttg ctcaattcaa catctcttgg gatgaaataa
attatgatcc tgaaggtaac gaaattgttc aacataaaaa ctggagcgaa aacaataaaa
gcaagttagc tcatttcaca tcgtccatct atttgccagg taacgcaaga aatattaatg
tttacgctaa agaatgcact ggtttagctt gggaatggtg gagaacggta attgatgacc
ggaacctacc gcttgtgaaa aatagaaata tctccatctg gggcactaca ctttatccga
aatatagtaa tagtgtagat aatccaatcg aataatttta aaaattaata aaaaaattaa
gaataaaacc gcttaacaca cacacg

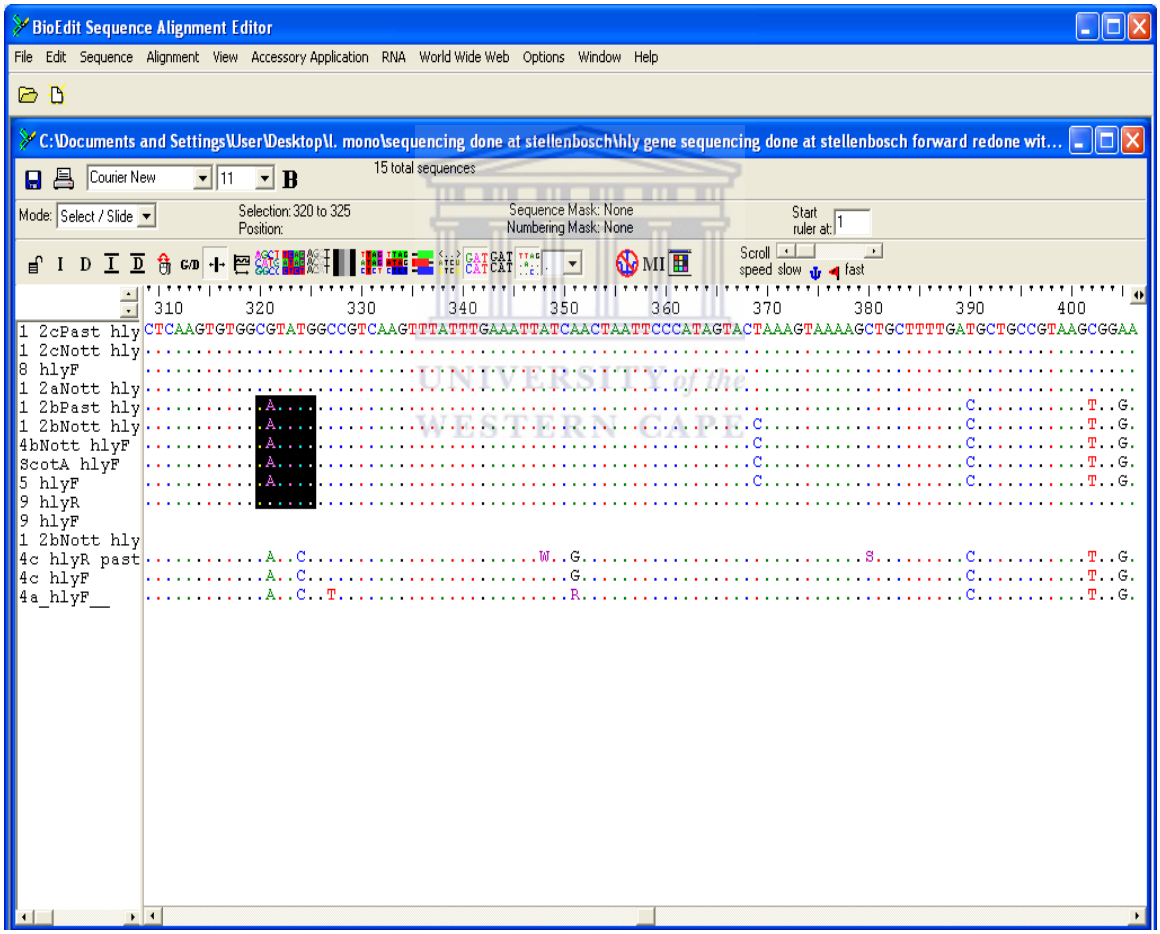
## Annexure 2

The sequencing results of *L. monocytogenes* isolates showing conserved SNP regions shared amongst lineage groups. For lineage II isolates, the enzyme recognition site including the SNP is highlighted in the box.



## Annexure 3

The sequencing results of *L. monocytogenes* isolates showing conserved SNP regions shared amongst lineage groups. For lineage I isolates, the enzyme recognition site including the SNP is highlighted in the box.



## Annexure 4

The sequencing results of *L. monocytogenes* isolates showing conserved SNP regions shared amongst lineage groups. For lineage III isolates, the enzyme recognition site including the SNP is highlighted in the box