AN ASSESSMENT OF THE VIRULENCE OF *LISTERIA* SPP. ISOLATED FROM RAW MILK AND RAW CHICKEN

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I DECLARE " AN ASSESSMENT OF VIRULENCE OF *LISTERIA* SPP. ISOLATED FROM RAW MILK AND RAW CHICKEN " IS MY OWN WORK AND THAT ALL THE SOURCES I HAVE USED OR QUOTED HAVE BEEN INDICATED AND ACKNOWLEDGED BY MEANS OF COMPLETE REFERENCES

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ABSTRACT

Listeria a foodborne pathogen is the etiological agent of the deadly disease listeriosis. Although, the virulence traits of international isolates of *Listeria* are well studied, nothing is known about the virulence traits of *Listeria* found in raw milk and raw chicken in the Western Cape, South Africa. Therefore, the aim of this study was to isolate *Listeria* from raw milk and raw chicken in the Western Cape, South Africa, in order to determine the virulence nature of such *Listeria* isolates. Two raw milk samples, collected from bulk tanks were analyzed for *Listeria* by the United States of America's Department of Agriculture Food Sanitation Inspective Services (FSIS) and the Food and Drug Administration (FDA) methods. Three *Listeria* species: *Listeria monocytogenes* (isolates DB121 and SK135), *L.grayi* (SK133) and *L.innocua* (SK118) were isolated from 2 raw milk samples and were identified by morphological and biochemical tests as well as by the *Listeria* API identification system. Eighteen raw chicken samples were analyzed by a modified FSIS method and 8 isolates AL1, AL2, AL3, AL5, L31, L35, P73 and P75 were identified as *L.monocytogenes* using the *Listeria* API identification system.

The virulence nature of the four *Listeria* isolates from raw milk and eight *Listeria* isolates from raw chicken were determined by the CAMP reaction, rhamnose and xylose acidification (CRX) tests, detection of a 60 kDa α-LLO toxin by sodium dodecyl sulphate polyacrylamide gel electrophoresis, Western blotting and immunoblotting and by oligonucleotide probing to detect the presence of the hly A gene. Six isolates were CRX positive and expressed a 60 kDa protein that gave a positive signal when reacted with anti-α-LLO antibodies. Genomic DNA samples of 5 of the 6 isolates also showed strong hybridization signals when reacted with hly A oligonucleotide probes. There was 91.7 % (11 of the 12 isolates) correlation between CRX phenotype, presence of a 60 kDa protein and the detection of the hly A gene.

UITTREKSEL

Listeria 'n voedseloordraagbare patogeen is die etiologiese agent van die dodelike siekte listeriose. Nieteenstaande die feit dat die virulensie van internasionale *Listeria* isolate goed bestudeer is, is daar huidiglik niks bekend omtrent die virulensie van *Listeria* in rou melk and rou hoender in die Wes-Kaap in Suid Afrika nie. Daarom was die doel van die studie om *Listeria* te isoleer vanuit rou melk and rou hoender in the Wes-Kaap, sodat die virulensie van die geisoleerde *Listeria* isolate bepaal kon word. Twee rou melk monsters vanuit twee melk tenks was geanaliseer vir *Listeria* volgens die United States of America's Department of Agriculture Food Sanitation Inspective Services (FSIS) en die Food and Drug Administration (FDA) metodes. Drie *Listeria* species: *L. monocytogenes* (isolate DB121 en SK135), *L.grayi* (SK133) en *L.innocua* (SK118) was geisoleer vanuit die twee rou melk monsters en was positief geïdentifiseer d.m.v van morfologiese en biochemiese toetse sovel as d.m.v die *Listeria* API identifikasie sisteem. Agtien rou hoender monsters was geanaliseer volgens 'n gemodifiseerde FSIS metode en agt isolate AL1, AL2, AL3, AL5, L31, L35, P73, en P75 was van rou hoender vleis geisoleer en positief geïdentifiseer as *L.monocytogenes* d.m.v *Listeria* API identifikasie sisteem.

Die virulensie eienskappe van vier *Listeria* isolate vanuit rou melk en agt *Listeria* isolate vanuit rou hoender was bepaal d.m.v die CAMP reaksie, rhamnose en xilose fermentasie toetse, waarneming van die 60 kDa, α-LLO Listeriolisien O toksien m.b.v natrium dodesiel sulfaat polyakrielamied gel elektroforese, Western blot klad tegniek en immunoblot en d.m.v. oligonukleotied peiler om die teenwoordigheid van die hly A geen te bepaal. Ses isolate was CRX positief en het 'n 60 kDa proteien gesintetiseer wat positief gereageer het met anti-α-LLO anti-liggame. Genomiese DNS monsters van 5 uit die 6 isolate het ook sterk hibridisasie reaksies vertoon toe dit gepeil was met hly A oligonukleotied peiler. Daar was 91.7% (11 van die 12 isolate) korrelasie tussen CRX fenotipe, die teenwoordigheid van 'n 60 kDa proteien en die waarneming van die hly A geen.

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1 GENERAL INTRODUCTION AND LITERATURE REVIEW

Food sustains life because it supplies humans with essential nutrients, but is equally capable of supporting the growth of a diversity of micro-organisms (98). Some micro-organisms form part of the natural microbial flora of some foods, whilst others come into contact with foods due to contamination from various sources (examples, air, soil, water, animals and human handling) (74). Some micro-organisms cause decomposition and spoilage of foods with little or no threat to human health, whilst others are potentially life threatening (74). It is then self-evident that a study of micro-organisms associated with food and their control is of primary importance.

Listeria causes a life threatening disease, generically termed listeriosis (151) Some of the most serious clinical manifestations of listeriosis are meningitis, abortions, encephalitis, septicaemia, pneumonic and respiratory problems (54, 142). Death commonly results from listeric meningitis if the infected individual is not treated (105). Listeric abortions occur in the last half of pregnancy and result in the birth of stillborn or acutely ill infants with pneumonic and respiratory listeriosis who often die within minutes or hours after birth, and an infant with listeriosis that is born alive, usually develops meningitis which results in death or permanent mental deficiencies (147, 151, 143). Individuals that are at great risk are the elderly (>60 years of age), pregnant women, infants, alcoholics, immuno-deficient persons and those with underlying diseases (7, 23, 62, 120). Therefore, an increase in listeriosis mortality may be expected among people with an underlying disease like AIDS (80). In healthy individuals, a self-limiting infection occurs without any serious health problems (54).

Listeria species were isolated as part of the natural microbial flora of soil, water (46, 170, 171) various avian species, food and wild as well as domesticated animals (16). The ability of *Listeria* species to survive in these environments suggest that these bacteria are well adapted to adverse conditions because soil, water, animals and food differ in extreme compositions, pH and temperature (50). This is supported by Silliker's claims, made at an international symposium (151), that *Listeria monocytogenes* has a survival capacity greater than other recorded foodborne pathogen and therefore, has the potential to cause widespread outbreaks with a high mortality rate, among susceptible individuals. This claim is substantiated by statistics that show an alarming international increase in listeriosis mortality since 1981 (42, 48, 68, 96, 97). Therefore, there is an urgent need to study all aspects of this micro-organism in order to control it effectively.

1.1 HISTORY OF TAXONOMIC CLASSIFICATION OF *LISTERIA MONOCYTOGENES*

In 1919, a bacterium was isolated from the necrotic foci of rabbit liver and identified as *Bacillus hepatis* (54). In the same year, French clinicians preserved an "unknown diphtheroid" which was isolated from the spinal fluid of a patient with meningitis and these cultures were so well preserved that in 1942 these cultures were identified as *Listeria monocytogenes* (54). In 1923, a bacterium was isolated from laboratory rabbits in England and named *Bacterium monocytogenes* because of the monocytosis it caused in the rabbits (54). The bacterium was first described in South Africa by Pirie in 1938 (122). This bacterium caused widespread death in rodents in the Orange Free State. He named the bacterium *Listerella hepatolytica*.

In 1959 Nyfeldt (116) isolated the etiological agent responsible for infectious mononucleosis and named it *Bacterium monocytogenes homonis*. According

to Gellin *et al.* (54), the mononucleosis that Nyfeldt observed in his patient was not a common feature of human illness associated with this bacterium. In 1940 the bacterium was officially named *Listeria monocytogenes* in honour of Lord Lister, an English surgeon, who is regarded as the father of antisepsis (145).

L.monocytogenes is a Gram-positive, non-spore-forming, facultatively anaerobic, rod-shaped bacterium that grows between -4.0°C and 50°C and is well described in Bergey's manual (145). *L.monocytogenes* was initially classified under the family Corynebacteriaceae (148), but was recently re-grouped with *Lactobacillus, Erysipelothrix, Brochothrix,* and other genera, in a section entitled "Regular Nonsporing Gram-positive Rods" (145). Currently, there are eight species classified under the genus *Listeria: L.monocytogenes, L.ivanovii, L.seeligeri, L.innocua, L.grayi, L.murrayi* and *L.welshimeri* (78).

1.2 *LISTERIA* IN SOUTH AFRICA COMPARED TO EUROPE AND AMERICA

Since 1981 there was an alarming increase in the number of reported cases of foodborne associated listeriosis outbreaks in Europe and America (10, 22, 48, 68) to the extent that it was referred to as "*Listeria* Hysteria", which underlines the fears and concerns of food manufacturers and consumers. Most of the outbreaks were marked by a high mortality rate (Table 1) and even more alarming is the world-wide increase in incidence of listeriosis (Table 2).

Tables 1 and 2 clearly indicate that *Listeria* is an international problem. An outbreak of listeriosis occurred in Johannesburg in 1978 (73). Fourteen cases were reported in nine months. It was the largest epidemic of listeriosis in South Africa, but there were no fatalities (73).

TABLE 1. Foodborne outbreaks due to *L.monocytogenes* (modified from Farber and Peterkin, 1991 (43))

Location(year)	Number of cases(no. deaths)	Food association
New Zealand(1980)	29(9)	shellfish, raw fish
Maritime Provinces of Canada(1981)	41(17)	coleslaw
Massachusetts(1983)	49(14)	pasteurized milk
California(1985)	142(48)	Jalisco cheese
Switzerland(1983-1987)	122(31)	raw milk, cheese
Philadelphia(1986-1987)	36(16)	ice cream, salami
Connecticut(1989)	9(1)	shrimp
United Kingdom(1987-1989)	>300 (unknown)	pate

TABLE 2. Incidence of listeriosis worldwide (modified from Farber and Peterkin, 1991 (43))

Country	Year(no. of cases)	incidence/million population
USA	about 1600 annually	8.3
Canada	1988(60)	2.3
Australia	1989(13)	7.6
New Zealand	1989(21)	7.0
Belgium	1989(48)	4.8
Denmark	1987(27)	4.7-5.3
	1989(32)	6.0
Finland	1989(29)	5.9
France	1984(630)	11.3
	1986(811)	14.7
	1989(416)	8.0
Norway	1989(7)	1.6
Scotland	1987(40)	7.0
	1989(29)	5.7
Switzerland	1989(32)	5.0
UK	1989(32)	4.3
Germany	1989(14)	5.8
Yugoslavia	1989(29)	3.0

Furthermore, the source from which individuals became infected was not known. Subsequent to the listeriosis outbreak that occurred in 1978 (73), sporadic isolated cases involving single individuals were also reported (1, 126 and S. Naidoo; pers. comm.[1]) and about 2-5 cases of listeriosis are annually reported in Soweto (H.J. Koornhof; pers. comm.[2]). From these reports it appears that listeriosis is not a major problem in South Africa as compared to listeriosis in Europe and America.

The reasons for the sparseness of listeriosis in South Africa may be due to: Firstly, confusion in diagnosis, because the initial symptoms of listeriosis are similar to those of influenza, therefore the possibility of incorrect diagnosis as a viral infection exists (42); secondly, no research has been done to assess the *Listeria* problem on a national scale; thirdly, routine industrial, microbial-safety-screening tests of foods are done using standard media for example liver-egg yolk agar for *Clostridium botulinum* or Lauryl sulphate tryptose broth and brilliant green lactose bile broth for coliforms (74) and since *Listeria* requires more complex media and enrichment procedures (99), it probably goes undetected.

1.3 ASSOCIATION OF *LISTERIA* WITH FOODS

1.3.1 Dairy products

Dairy products were implicated in most of the foodborne listeriosis outbreaks and are the cause of a high incidence of listeriosis world-wide (38,

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2 H.J. Koornhof, Medical Reseach Council, Private Bag X385, 0001 Pretoria, South Africa.

47, 48, 97). The overall worldwide incidence of *L.monocytogenes* in raw milk is reported to be approximately 2.2% (43).

In 1983 an outbreak occurred in Massachusetts in which 42 people were infected and 14 mortalities occurred (48). The outbreak was associated with the drinking of a specific brand of pasteurized milk. The raw milk came from a group of farms where bovine listeriosis was known to have occurred at the time of the outbreak. There was no evidence of improper pasteurization at the plant (48) implying that *Listeria* survived the pasteurization process.

In 1985, an outbreak of listeriosis occurred in California (97). This was one of the most severe outbreaks in which 142 cases were reported and a total of 48 deaths occurred. The consumption of contaminated Mexican-style soft cheese was implicated by case-control studies (97). All of the 142 people had eaten the cheese and *L.monocytogenes* type 4b was isolated from both the individuals and the samples taken from the plant producing the cheese. No *Listeria* was found in the raw milk samples taken from the dairy farms which supplied the cheese manufacturing plant and no evidence of improper pasteurization was found (97).

From 1983-1987, 122 listeriosis cases were reported in Switzerland and 31 deaths were recorded (22). Jalisco cheese and Vacherin Mont d'Or soft cheese were implicated (22).

From 176 raw milk samples tested incidence of 10.2%, 2.8% and 27% were reported for *L.innocua*, *L.seeligeri* and *L.monocytogenes*, respectively and none of the other *Listeria* species were isolated (65). Moreover, from 110 raw milk samples the following *Listeria* spp. were isolated and the numbers in brackets indicate the number of samples from which a particular species was isolated, *L.grayi* (3), *L.innocua* (10), *L.seeligeri* (6), *L.welshimeri* (6) and

L.monocytogenes from 13 samples (133). From a total of 982 raw milk samples taken in the Transvaal, South Africa, 81% of the raw milk from dairy silos contained *L.monocytogenes* (174).

From 22 fresh Jben cheese samples tested, *L.innocua* was isolated from four samples and 13 samples contained *L.monocytogenes* (104). Incidence of *L.seeligeri* was 0.03% in 33 soft cheese samples that were tested, whereas *L.monocytogenes* had a 5.2% incidence (65). From 20 samples of butter, tested only 5% contained *L.innocua* and none of the other *Listeria* species were isolated (106). In some cheeses, *L.monocytogenes* levels of 10^7 colony forming units per gram were reported (135).

Although a lower incidence of *L.monocytogenes* is expected in pasteurized milk, one report indicated an increase of this bacterium in pasteurized milk taken from one Madrid dairy plant (47). Other dairy products from which *L.monocytogenes* were isolated included: ice-cream (4), yoghurt, buttermilk, soyamilk and skim milk (65, 134).

Infected cows may shed *L.monocytogenes* in milk at populations of 10^3 cells/ml; this was suggested as a possible reason why dairy products are more susceptible to contamination (16). Moreover, cows might be infected through the consumption of contaminated water and silage because *Listeria* is part of the natural microbial flora of these ecological sites (16). *Listeria* populations greater than 1.2×10^4 cells/g of silage have been reported (46). Furthermore, *L.monocytogenes* may persist in silage for 10-12 years and it was suggested that silage might be the primary reservoir from which cows become infected (46).

The economic impact that *L.monocytogenes* might have on the dairy industry is immense. During the listeriosis outbreak in which 48 people died from the

consumption of *Listeria*-contaminated Mexican style soft cheese, the factory which manufactured the cheese was closed and all implicated cheese was recalled (97). Secondly, when *L.monocytogenes* was detected in Polar B'ar ice-cream during routine quality control testing the company voluntarily recalled all Polar B'ar ice-creams (43). No details of the cost are mentioned, but if cost analysis are made according to Todd (161) then it is self-evident and that financial losses were suffered by the two factories in the following areas: from loss productivity when the factory had to be closed and cleaned; recall of all implicated products; legal settlements, medical cost and the loss of human life. Moreover, all these factors will impact negatively on a company's image and will affect future sales. Although no details were supplied about the specific number of each type of dairy product that was recalled, Mattingly *et al.* (107) states that 500 dairy products contaminated with *Listeria* were recalled between the period 1987-1988 in the USA at a total loss of 70 million US dollars. This amount clearly illustrates the enormous economic impact that *L.monocytogenes* might have on the dairy industry.

1.3.2 Meat products

Although *L.monocytogenes* has been found in a wide variety of meats (40, 63, 77, 112), no epidemic listeriosis outbreaks occurred from the consumption of contaminated meats (43). There is only one well-documented case implicating meat (turkey frankfurters) in a sporadic incident of listeriosis (10). Although only one woman became ill from eating turkey frankfurters, 6 million lb of turkey hot dogs were recalled (10). The numbers and serotypes of *Listeria* species present in meat vary and most of the contamination occurs on the surface of the meat probably indicating external contamination from processing equipment and meat handlers (43). However, *L.monocytogenes*

was recently isolated from the interior muscles core of beef, pork and lamb (77).

The most prominent serotype found in meats is *L.monocytogenes* serotype 1, but recently serotype 4b has been isolated from pate during a survey (1987-1989) in the United Kingdom (112). Other meat products from which *L.monocytogenes* were isolated include: frozen-and-ready to cook chicken (59, 121); processed meat (9, 164, 166) beef (3, 163) lamb (43) and pork (19). From 342 samples of processed meats tested the following incidence was reported: *L.monocytogenes* (13.2%), *L.innocua* (24%), *L.welshimeri* (1.2%) and *L.seeligeri* (0.9%) (164). An incidence of 32.2% for *L.innocua* and an incidence of 11.3% for *L.monocytogenes* have been found in 292 samples of various types of meat (19). Raw sausages had the highest incidence (51%) of *L.innocua* from 102 samples that were tested (19).

The highest contamination with *L.monocytogenes* was found in chicken. Pini (121) found that 66% of 50 fresh chicken samples and 54% of 50 frozen chicken samples were contaminated with *L.monocytogenes*. From 30 samples of oven ready poultry samples tested 50% were contaminated with *L.monocytogenes* and 10 samples were contaminated with *L.innocua* (70). It was also reported that from 35 retail fresh and frozen chicken samples, 57% contained *Listeria* (90). *Listeria* was also isolated from two cooked-chill-ready to eat chicken samples (total sample size was 21) and each of the two samples contained 400 *L.monocytogenes* cells per gram (83).

The source of contamination of meat by *Listeria* is not known, but since *L.monocytogenes* was isolated as part of the natural microbial flora of cattle, sheep, chickens and various other avian species (16) it is likely that contamination occurs from animals which harbour *Listeria*. Although meat is widely contaminated with *Listeria* no epidemic listeriosis occurred as the result

of the consumption of contaminated meat. This is probably because *L.monocytogenes* serotype 1 occurs most frequently in meat and is less virulent, whereas the more virulent *L.monocytogenes* serotype 4b occurs most frequently in dairy products (43). Another non-virulent *Listeria* species, *L.innocua* was predominately found in various meats (19).

1.3.3 Egg products

L.monocytogenes occurs in low numbers (1-8 colony forming units per ml) of commercially broken raw liquid eggs (92) and it has been demonstrated that *L.monocytogenes* was unable to grow in artificially inoculated whole eggs stored at 5°C for 22 days (153). No listeriosis outbreaks have occurred which implicated egg products.

1.3.4 Vegetables and fruits

In Boston in 1979 an outbreak of listeriosis occurred in which 20 people became ill and 5 died (68). Raw celery, tomatoes and lettuce were implicated. In 1981, in the Maritime Provinces of Canada, an outbreak of listeriosis involving 29 cases with 9 deaths occurred (135). Coleslaw was implicated. An epidemiological study indicated that the manure from animals infected with *L.monocytogenes* contaminated the soil which in turn contaminated the crop (43). Moreover, cultivated and uncultivated soil are natural reservoirs for *L.monocytogenes* (16).

Many different types of vegetables and fruits have been screened for *Listeria*, but only potatoes and radishes appear to be regularly contaminated (67). From 1000 samples of fresh raw vegetables tested *L.monocytogenes* had an incidence of 43% and occurred predominately in potatoes and radish (67). The incidence of other *Listeria* spp. was as follows: *L.welshimeri* (3%),

L.seeligeri (13%) and *L.innocua* (24%) (67). No *Listeria* spp. were isolated from broccoli, carrots, cauliflower and tomatoes (67). From 60 samples of mixed fruit and vegetable salads tested, 2 samples contained *L.monocytogenes* and 13 samples were contaminated with *L.innocua* (154). Moreover, *L.innocua* numbers increased two fold when samples containing this bacterium was stored at 4°C for 4 days (154). No outbreak of listeriosis was ever reported which implicated potatoes, radishes or fruits.

1.3.5 Seafood

Since *L.monocytogenes* has been isolated as part of the natural microbial flora of the surface waters of rivers, lakes and canals (16, 167), it is highly probable that fish and other water animals might be contaminated with this bacterium. Many fish products in North America including frozen and cooked shrimp, canned, frozen crab meat, smoked salmon and frozen lobster were contaminated (168). Fish and related products were implicated in two listeriosis outbreaks. In 1980, in New Zealand, 29 cases with 9 deaths occurred in which shellfish and raw fish were implicated (96). Also, in Connecticut, in 1989, an outbreak of listeriosis involving 9 cases with one death occurred and shrimp was implicated (43). Dried-salted fish was free of *L.monocytogenes*, but three out of the ten samples contained *L.innocua*, moreover, five out of fourteen frozen fish samples also contained *L.innocua* (51).

In summary, *Listeria* occurs widely in a variety of foods and it appears that most processing and preservation methods such as pasteurization, freezing, cooking and chemical additives are generally not effective for the successful elimination and/or control of *Listeria* in food such as dairy products, meat, fish, vegetables and fruits. Therefore, the association of *Listeria* with foods has become a major concern to food manufacturers and consumers, especially

considering the damaging effect this bacterium could have on human health and on the economy.

1.4 EFFECT OF FOOD PROCESSING AND PRESERVATION ON *LISTERIA MONOCYTOGENES*

There are various methods to process and preserve foods (74, 119). These methods ensure that the final product is free of most infectious microbial pathogens, their harmful enzymes and toxins; and also reduce the number of spoilage micro-organisms to an acceptable minimum level and hence the product is made safe for human consumption (74, 119).

We will review the effects that some of the most commonly used methods such as pasteurization, refrigeration and the addition of selected antimicrobial chemicals have on eliminating and/or controlling of *Listeria* in foods. Since *L.monocytogenes* was the etiological agent in all the listeriosis outbreaks (Table 1) and this bacterium is considered a major foodborne pathogen for humans (54), whereas the other *Listeria* spp. are generally considered to be non-pathogenic for humans (130, 137) therefore, it is obvious that most research on the elimination and/or control of *Listeria* in food has focused on *L.monocytogenes.*

1.4.1 Effect of refrigeration

One of the most commonly used methods of food preservation is refrigeration (74). Most foods are stored at either one of the following three low temperatures: chilling temperature (10°C-15°C) or refrigeration temperature (0-2°C or 5-7°C) or freezing temperature (-18°C and below) (74). Low temperatures exert their effect by inhibiting metabolic enzymes which are

essential for bacterial growth (74). The microbial numbers immediately before refrigeration and/or freezing may gradually decline due to mortality upon prolonged storage at extreme low temperatures (74).

L.monocytogenes growth rate is 13-25 colony forming units per hour at 0-1°C and 162-131 colony forming units per hour at 4-5°C and true psychrophiles have growth rates of 6 colony forming units per hour at 0-1°C and 12 colony forming units per hour at 4-5°C; whilst psychrotrophic Enterobacteriaceae and spoilage bacteria have growth rates of 8-12 colony forming units per hour and 16-20 colony forming units per hour at the same temperatures, respectively (81). Therefore, it appears with the exception of *Salmonella* spp. that *L.monocytogenes* can outgrow most psychrotrophic and psychrophilic bacteria at these temperatures (81). Growth greater than 30 colony forming units per hour was recorded at (4-5°C) and (0-1°C) for *Salmonella* species (81).

A listeriosis outbreak occurred in Philadelphia (1986-1987), in which 16 people died out of the 36 cases that were reported (140). This was due to consumption of ice-cream which is normally stored at temperatures below -20°C (140). Moreover, viable *Listeria* was isolated from several types of frozen products: beef patties (43), chickens (121), ice-cream (4) and seafood (168). From these reports it appears that *L.monocytogenes* could also survive at temperatures below -20°C.

1.4.2 Effect of pasteurization

Pasteurization is a process whereby food, especially milk is heated for a specific time in order to kill most infectious human pathogens and also to inhibit the growth and reduce the number of spoilage micro-organisms to an acceptable maximum level (119). The end product is thus made safe for human consumption. Three pasteurization methods are being used, mainly:

low-temperature, long-time hold, (62.8°C, 30 minutes (LTH)); high-temperature, short-time, (71.7°C, 15 seconds (HTST)) and ultra-high temperature (140-150°C for 2-5 seconds (UHT)) (119). These processes, unlike sterilization, do not kill all types of micro-organisms or spores, but are effective in eliminating human pathogens (119). Therefore, it was alarming when two outbreaks of listeriosis associated with the consumption of LTH and HTST milk were reported (48). No evidence was found to suggest improper pasteurization, or post-pasteurization handling contamination. Due to these two outbreaks (48), studies were conducted into the thermal resistance of *L.monocytogenes* in order to assess the effectiveness of pasteurization against this bacterium (18, 44, 88, 108). There are many contradictions with regard to the effectiveness of both LTH and HTST to eliminate *L.monocytogenes* from milk. Some reports indicate that *L.monocytogenes* can survive HTST pasteurization, whereas others indicate that it cannot survive this treatment. One study showed that pasteurization of inoculated raw milk in a pilot plant-size pasteurizer, at temperatures ranging from 69-73°C for 15 seconds, was ineffective as *L.monocytogenes* was isolated from samples after 2 days to 3 weeks (47). Another study (18), conducted over a two year period, determined the decimal reduction time (D-value) of *L.monocytogenes* Scott A strain and found that this strain suspended in raw milk and heat treated for 71.7°C for 15 seconds in sealed glass tubes was unable to survive this treatment and this led to the conclusion that the HTST pasteurization process was effective.

To explain these contradictions it was suggested that since *L.monocytogenes* grows intracellularly, and certain cells (eg. phagocytes) in milk could afford the bacterium additional thermal resistances (44, 102). Another suggestion was that *L.monocytogenes* grown at high temperatures greater than 43°C for 5, 30 or 60 minutes before D-value determinations was more thermotolerant than cells grown at lower temperatures because of the heat shock phenomenon

(88). Farber *et al.* (44) found that *Listeria* strains grown at 43, 39 and 30°C on tryptose agar or broth and then inoculated in to raw milk and heat treated at 72°C for 16 seconds in a pasteurizer, showed a decrease of 2.1, 2.8, 4.1 logs, at the respectively temperatures. Also, *L.monocytogenes* enumerated after heat treatment (62.8°C) showed a sixfold increase when cultivated under strict anaerobic conditions (43°C) as opposed to cultivation under aerobic conditions at 37°C (88). Moreover, Doyle *et al.* (40) claim that the type of recovery media play an important role, because *L.monocytogenes* treated at 71.7-73.9°C for 16.4 seconds was only recovered by enrichment and not by direct plating. Better recovery at 4°C was obtained for *L.monocytogenes* using blood agar after heat treatment (58-62°C for 15-20 seconds) (156). No *Listeria* species were isolated from UHT processed milk (65), thus implying that *Listeria* cannot survive UHT pasteurization treatment.

Therefore, from the above reports, it appears that *Listeria* can survive LTH and HTST treatment and that the recovery media and incubation temperature and incubation conditions (areobic or anaerobic) must be considered when assessing growth after pasteurization treatment.

1.4.3 Effect of antimicrobial agents

Some commonly used antimicrobial food preservatives are sodium chloride, nitrite, nitrate and nisin (74). A discussion on these preservatives and their effectiveness against *L.monocytogenes* follows.

1.4.3.1 Sodium chloride

The use of NaCl to preserve meat and fish dates back to 3000 B.C. (74). The principal action of NaCl is that at high concentrations it dehydrates both the food and the micro-organism (74). A bacterium placed in an isotonic

salt solution (0.85-0.9% NaCl) will grow unaffected because the amount of NaCl and water is equal on both sides of the cell membrane of the bacterium and water diffuses equally in both directions, but if the salt is increased to about 5% or more (hypertonic solution) for bacteria which are not halophiles or halodurics (normally withstand about 20% NaCl) water will diffuse out of the bacterium cell through the process of osmosis at a greater rate than it would enter (74). The bacterium will undergo plasmolysis (cell shrinkage) and this will result in growth inhibition or even death (74). The food can also undergo shrinkage by a similar mechanism.

The second effect that NaCl has is to reduce the water requirements of the micro-organism (water activity (a_w)) (74). High NaCl salt concentration reduces a_w below the absolute minimum water requirement and therefore, the micro-organism cannot conduct certain essential metabolic processes and this in turn can inhibit growth (74). The a_w of most fresh foods is about 0.99 and most food spoilage bacteria do not grow below an a_w of 0.91 (achieved by about 15% NaCl) (74). *Staphylococcus aureus* is capable of growth at a_w 0.85-0.92 (20-13% NaCl) (74). *L.monocytogenes* has been shown to grow at a pH $\geq$ 5.0 in presence of 10% NaCl (a_w 0.93) (108). *L.monocytogenes* survived for one year in 16% NaCl (pH 6.0, 37°C) and for 5 days in 20-30% NaCl at 37°C (40). Longer survival times were noted when the temperature was lowered, for example, at 4°C the bacterium survived for 100 days in 10.5-30% NaCl (27).

1.4.3.2 Nisin

Nisin is one of two antibiotics permitted for use as a food preservatives in 39 countries (72). Of the two, nisin, rather than natamycin, is more commonly used for food preservation (74). Nisin has the following desirable properties as a food preservative: it is effective against Gram positive bacteria

(primarily sporeforming bacteria) and relatively ineffective against Gram negative bacteria, it is not used medically and is completely destroyed in the human gut (74). Natamycin is only effective against fungi and is also used in animal feeds (74). The international permissible level of nisin in foods is between 2.5-100 international units (IU) depending on the food (74). These levels were found to sufficiently inhibit the growth of human pathogens and food spoilage bacteria and even the spores of these bacteria (74). One report noted that 2000 IU of nisin per ml of tryptose broth at 37°C was not sufficient to inhibit the growth *L.monocytogenes* Scott A serotype, whereas another serotype of *L.monocytogenes* was inhibited by 16-32 IU (40). This could imply that resistance to nisin varies between different serotypes of *L.monocytogenes* (40).

1.4.3.3 Nitrite and nitrate

Sodium nitrate ($NaNO_3$) and sodium nitrite ($NaNO_2$) are used for curing, colour stabilization and flavour development in red meats, but also inhibit growth of spoilage and pathogenic micro-organisms (74). Certain bacteria are able to use nitrate as an electron acceptor and in the process reduces it to nitrite. In an acidic environment, nitrite ionizes to nitrous acid ($3HNO_2$) which further decomposes to nitric oxide (NO) (74). Nitric oxide affects various porphyrin-containing components such as catalase and cytochromes. Nitrate and nitrite is effective against *S.aureus* and ineffective against Enterobacteriaceae (74). *L.monocytogenes* was not inhibited in meat by permitted levels (100 - 200 IU) of nitrite, except when a combination of nitrite plus NaCl was used (eg. 100 IU nitrite, 3% NaCl, pH < 5.5) (40).

Generally it appears that the effectiveness of NaCl, nisin, nitrite and nitrate as antimicrobial agents against *L.monocytogenes* is dependent on a number of

interrelated factors such as temperature, pH and concentration. In addition resistance to nisin varies between different serotypes of *L.monocytogenes*. Therefore, all these factors need to be considered when using a particular antimicrobial agent against *L.monocytogenes*. Although, nothing is known about the effects of these preservatives on other *Listeria* species, it could be speculated that sodium chloride would have the same effects on other *Listeria* species, but that nisin and nitrate might have a different effect on other *Listeria* species because the cell wall and cell membrane chemical composition of these other *Listeria* species are different from *L.monocytogenes* (145).

1.5 PATHOGENICITY OF *LISTERIA* SPECIES

Of the seven *Listeria* species, *L.monocytogenes* is considered to be the major pathogen of humans because all listeriosis outbreaks thus far were due to *L.monocytogenes* (43). Related species occur as innocuous saprophytes in the environment (107), except for *L.ivanovii* which is a major pathogen of animals (95). There is only one report that indicated that *L.ivanovii* caused human listeriosis in isolated cases (147). *L.seeligeri* is generally considered to be non-pathogenic. However, it has been implicated in one case of human listeriosis (95). This case involved an adult male suffering from meningitis. It was proven that *L.seeligeri* was the etiological agent since it was the only organism isolated from the cerebrospinal fluid of the sufferer (95). Apart from these two reports implicating *L.ivanovii* and *L.seeligeri* in human listeriosis, no other reports have made mention of human listeriosis related to these bacteria and consequently they are not generally considered to be major human pathogens. *L.welshimeri, L.grayi, L.murrayi* and *L.innocua* are reported to be non-hemolytic and non-pathogenic (137, 139, 146).

There are 16 serotypes of *L.monocytogenes*. Only four serotypes (4b, 1/2b, 3b and 1/2a) are reported to be responsible for more than 90% of all human listeriosis (54, 139). Since *L.monocytogenes* is considered to be the major human pathogen compared to other *Listeria* species therefore, the ensuing discussion will focus on the virulence of *L.monocytogenes*.

There are two types of epidemiological listeriosis: epidemic listeriosis (characterized by its occurrence at a specific time, distribution among a specific population and linked to specific contaminated foods) and sporadic listeriosis (disease has no recognized epidemiological pattern, it appears occasionally and may be influenced by seasonal changes where the cases are normally isolated and never in a specific cluster which can be traced to a common cause) (43). Epidemic listeriosis appears more frequently and is responsible for the high mortality rate (22, 23, 101). Since the outbreaks of 1981 where listeriosis was conclusively linked to the consumption of particular foods, *L.monocytogenes* has been considered an important foodborne pathogen (138). This led to the suggestion that *L.monocytogenes* enters the human body *via* the oral route. This implied that *L.monocytogenes* could only gain entry into the body *via* the consumption of contaminated food or water and then cause an infection, hence the term foodborne pathogen (105). Foods, in turn, may become contaminated from various sources such as soil, air, water, animals and even human food handlers (16).

The first proof, that indicated that soil can contaminate foods was obtained by a study that traced the cause of an outbreak which was associated with the consumption of coleslaw (138). The cold-stored cabbage used in the coleslaw contained *L.monocytogenes*, furthermore the soil on which the cabbage was grown also contained the same infectious serovar of *L.monocytogenes*. It was then argued that the storage of the soil

contaminated cabbage at this low temperature, selected for the growth of *L.monocytogenes* (138).

Moreover, vegetables, fruit and animals harbour *L.monocytogenes* as part of their natural microbial flora, thus it is reasonable to assume that when foods are inadequately processed and/or handled and/or preserved (as seen from section 1.4), *Listeria* will survive and human consumption of these foods may cause listeriosis (22, 40).

Although the major route of entry of *L.monocytogenes* into the human body is the oral route, nevertheless direct transmission of this bacterium from infected animals to humans is possible (105). Several cases are mentioned where farmers and farm-helpers became ill shortly after they worked with diseased animals (105). Direct transmission of *L.monocytogenes* from ticks and flies to humans has also been reported (5). There is also a highly controversial case of *L.monocytogenes* transmission by air to humans (105). This case involved a Norwegian farmer who became ill while cleaning his sheep stables. It is postulated that he inhaled the air, in which the bacterium became suspended, during the cleaning operations and became ill (105).

In conclusion it appears that the main route of transmission for *L.monocytogenes* is *via* food consumption, but other modes of transmission are possible. However, only foods have been associated with epidemic listeriosis (43).

It has also been found that *L.monocytogenes* formed part of the normal gut microbial flora of an insignificant number (9 out of 1732) of healthy individuals that were surveyed (54). These individuals never became ill. The reason for this is probably because the type of serovars which were found in their gut might have been less virulent than previously identified strains. Only

L.monocytogenes serovars 1/2a, 1/2b, 4b and 3b are highly virulent and infectious (54, 137). Also, the number of *L.monocytogenes* in the gut might be too low to constitute a minimal infectious dosage (MID) owing to competition with other gut bacteria (81). The MID parameter is difficult to determine because it is affected by: genotypic and phenotypic virulence attributes of the pathogenic species; the health status of the individual at risk; modes of ingestion (eg. on an empty stomach or not); the type and amount of food eaten and the normal microbial flora within the gut (81).

It was found that a dosage of 10^9 cells per 10-20 g mouse body weight, infected orally, resulted in illness of the mice and the symptoms mimicked human illness, these ranged from septicemia to meningitis and eventual death (158). It is difficult to extrapolate MID which was determined in animals to humans because humans are physiologically and metabolically more complex. Therefore, MID for humans are not known, although estimates from actual cases of individuals, naturally infected with *L.monocytogenes,* are 10^2-10^4 cells per 75-100 kg human body weight (97), while another report indicates a MID of 3.4 x 10^9 cells per 75-100 kg human body weight (5).

1.5.1 Virulence and virulence factors of *Listeria*

Pathogenicity (capacity to cause disease) is not a common characteristic among micro-organisms (30). Many pathogens exhibit organotropism (affinity for specific tissues or organs). Most pathogens penetrate (invasiveness) the membrane of a particular cell and are internalized (30). Some pathogens are capable of intracellular growth, causing disruption of the normal physiological processes of the host, whilst other pathogens grow extracellularly causing damage to the cells by toxins (157). The pathogenicity of bacteria is determined by three factors: the dosage (minimal infectious dosage), the phase of growth (be it stationary or exponential) and most importantly by the

type of virulence factors (weapons) it possesses (30, 119). All of these will determine the degree of damage a particular bacterium may inflict on its host. The measured degree of pathogenicity and hence the degree of damage a pathogen can cause, is expressed as virulence (152). The presence of particular virulence factors can increase the pathogenicity of a particular micro-organism. Virulence factors range from the possession of a capsule to the production of specific factors, eg. toxins and/or enzymes under the infection process that causes disease development (157).

The problem arises how to determine virulence factor(s). Even more difficult is how to determine which of these virulence factor(s) cause significant damage to the host (157). In many cases the "final damage" (clinically observable symptoms) might involve a number of inter-related factors, including the host defence response and the synergistic co-operations of a micro-organism's different virulence factors with each other and with other factors (eg. various enzymes) (50).

Virulent strains of *L.monocytogenes* are postulated to have several different virulence factors (6, 55, 69, 118, 124, 172) and these will be discussed below, but it appears that the Listeriolysin O toxin is the most crucial virulence factor that plays an important role in listeriosis (11, 12, 24, 26, 28).

1.5.2 Intrinsic virulence factors of *L.monocytogenes*

1.5.2.1 Increased enzyme content

Virulent *L.monocytogenes* strains produce high amounts of superoxide dismutase and catalase intracellularly, compared to avirulent strains (32). These enzymes are postulated to play a role in the destruction of free radicals such as superoxides (O^{2-}) and H_2O_2 which are the main constituents of

phagocytes (60). Phagocytes are cells found in the human body involved in non-specific immune defence (30). Phagocytes contain different types of lysosomal enzymes and free radicals which may oxidize different components within the cell membrane of micro-organisms, eg. the SH-groups of certain proteins are destroyed by sequential free radical chain oxidation reactions (8). Superoxide dismutase is able to bind O^{2-} and catalase is able to split the harmful H_2O_2 resulting in the release of O_2 (60, 103, 169). The 50% lethal dose (LD_{50}) for mice was lowered in catalase producing *Listeria* strains (169). An *in vitro* study indicated that *L.monocytogenes* was resistant to oxidative antibacterial agents such as hydroxyl radicals, H_2O_2 and hypochlorous acid during log phase growth when the catalase concentration was high (15). However, no direct correlation could be established between intracellular survival of *L.monocytogenes* and increase production of superoxide dismutase (169). Therefore, catalase and superoxide dismutase act as free radical scavengers and hence prevent the destruction of *L.monocytogenes* by phagocytes (60) and this may contribute to the survival of the bacterium within the phagocytes.

1.5.2.2 Cell-wall structure

Certain virulent micro-organisms have clearly defined cellular structures eg. the capsule of *Klebsiella pneumoniae* which promote virulence (157). A variety of components have been found within cell wall extracts of *L.monocytogenes* which contain substances that contribute to the pathogenicity of the bacterium (105). These included carbohydrates and protein fractions which were antigenic, pyrogenic (pus-producing) and capable of inducing granulocytosis (proliferation of granulocytes) (105). Granulocytes are involved in non-specific immune defence in the human body (30). These fractions were not purified to homogeneity nor were they characterized, therefore, their involvement in pathogenicity is not yet known (105).

1.5.2.3 Toxins

It is mentioned that *Listeria* produces several types of toxins (147): hemolysins; an endotoxin-like lipopolysaccharide (LPS); an extracellular hemorrhagic toxin (toxin involved in the rupture of blood vessels); pyrogenic toxin; and an electrocardiographic toxin (a toxin that alters the heart beat rhythm). These toxins are only referred to by Seeliger and Finger, 1976 (147). No other report on these toxins were found, except for reports on LPS toxin (29, 172) and the hemolysin (Listeriolysin O toxin)(28, 57). Therefore, it is not clear whether each of these toxins, reported by Seeliger and Finger (147) are unique homogenous toxins, or whether the same toxin was eliciting the various observed reactions. It is possible that the same toxin produced the various reaction manifestations and these various reaction manifestations might have led to the report of different toxins (105). Hemolysin (LLO toxin) was proven to cause electrocardiographic changes (85, 86) and even the pyrogenic effect was observed for the LPS toxin (172). Also, the only two toxins that have been purified to homogenicity and which are well defined are the hemolysin (LLO toxin) (57) and the LPS toxin (172). Of the two toxins, only LLO toxin is widely reported to be an important virulence factor which is crucial for the pathogenicity of the bacterium (11, 12, 14, 26, 28, 52).

The presence of the LPS toxin was first illustrated when heat killed *L.monocytogenes* serotype 4b stimulated the production of cold agglutination (CA) antibodies in rabbits (29). These CA antibodies were shown to be responsible for the commonly observed cold agglutination syndrome (CAS) that is characterized by chills and flu-like symptoms. These symptoms are symptomatic of the initial clinical signs that are observed in listeriosis sufferers (105).

In 1976, Wexler and Oppenheim (172) isolated, purified and characterized the fraction responsible for the CAS. It was a fraction similar in composition to other known endotoxins, except that it was produced by a Gram-positive bacterium and was, therefore, termed an endotoxin-like factor (172) (classic definition stated that endotoxins are only produced by Gram-negative bacteria, hence the unusual name (119)). Intravenous injection of 10-400 μg purified LPS toxin into 2 kg rabbits, resulted in a pyrogenic response accompanied by malaise, severe diarrhoea and eventual death within 2-24 hours (172). An autopsy of rabbits which died within 2 hours, from a dosage of 50 μg, showed only gastrointestinal and stomach rupture symptoms (172). None of these histopathological symptoms are seen in humans (23, 54, 62, 101, 105) naturally infected with *L.monocytogenes* or rabbits infected with purified LLO toxin (86, 87). Human deaths from listeriosis is chiefly due to listeric meningitis and/or respiratory failure and/or abortion (151).

Therefore, it seems that the LPS toxin is only responsible for the initial flu-like symptoms observed in human listeriosis victims. Moreover, the lethality of the LPS toxin was 10-400 μg per 2 kg rabbit (172), whereas a dosage of 0.8 μg of purified LLO toxin per 10-20 g mouse body weight was lethal to mice (57). Since these studies were conducted in different animals it is difficult to compare and say which of the two toxins are more potent.

1.5.3 The relationship between β-hemolysis, Listeriolysin O and virulence

In 1932, Nyfeldt (116) observed that only *Listeria* species isolated from naturally infected individuals, produced β-hemolysis on sheep blood agar plates and killed mice (virulence test) (116). This led to the early assumption that β-hemolysis was correlated with virulence and implied that the factor responsible for β-hemolysis was the same factor responsible for pathogenicity (130). Moreover, it implied that observation of β-hemolysis was sufficient evidence

that a particular *Listeria* strain was pathogenic (69, 130, 155). Therefore, β-hemolysis was regarded as an excellent determinant and as a suitable marker that could be used to differentiate between pathogenic and non-pathogenic *Listeria* strains (69, 155).

The speculation that β-hemolysis correlated with virulence of *Listeria* was supported by experiments which showed that when β-hemolytic *Listeria* strains were artificially injected, the bacteria caused conjunctivitis (Anton's virulence test) in the eyes of rabbits, guinea-pigs and monkeys (130, 131). The conjunctivitis symptoms in animals corresponded to clinically observed conjunctivitis in humans, who became naturally infected with *Listeria* (69, 130). Further support for this assumption, came from the observation that radiation induced mutants of virulent, hemolytic *Listeria* strains yielded avirulent *Listeria* strains or *Listeria* strains with decreased virulence and the mutant *Listeria* strains were non-hemolytic and non-pathogenic (71).

The assumption that β-hemolysis correlated with virulence was challenged. Girard *et al.*, 1963 (58) observed the simultaneous occurrence of hemolytic and non-hemolytic *Listeria* colonies on blood agar plates and also observed variations in the morphology of the same colonies taken from a single hemolytic strain. This implied that hemolytic *Listeria* strains spontaneously mutated to non-hemolytic *Listeria* strains. Moreover, an increase in virulence of *Listeria* was correlated with a decrease in hemolysin production (114, 115). Another report stated that all *Listeria* strains (not only virulent strains) were capable of lysing red blood cells and that the degree of lysis (weak to unobservable) was influenced by the nutrient composition of the media that was used as underlay for blood and also influenced by the type of blood (sheep, horse and human blood) used (129, 131). Furthermore, Welshimer *et al.* (170) found that a *L.monocytogenes* strain that was lethal for mice failed to induce hemolysis on blood agar base containing 5% rabbit or sheep

blood. Different intensities (weak to strong) of hemolysis were observed for various *Listeria* spp. that were grown on sheep blood agar (139).

Ralovich *et al.* (130, 131) contested the experiments of Girard *et al.* (58) and of Njoku-obi *et al.* (114, 115), claiming that the sample size used was too small to draw any significant conclusions, and secondly, that no *in vivo* virulence tests were performed in parallel with hemolysis on blood agar.

In conclusion it appears that β-hemolysis correlates with virulence of *Listeria* strains, but that the composition of the media and the type of blood may influence hemolysis and make the use of hemolysis to differentiate between pathogenic and non-pathogenic *Listeria* strains unreliable (128, 154).

1.5.4 β-Hemolysis

Hemolysis is a complex process whereby a limited number of specific organic and inorganic molecules cause lysis (rupture) of red blood cell membrane, liberating haemoglobin (152). Hemolysis can be demonstrated *in vitro* in either a test tube or on blood agar plates (119). The agent which causes hemolysis is generally termed a hemolysin (152). When blood agar plates are treated with a hemolysin it will cause the erythrocytes to rupture resulting in a clearing zone around the hemolysin and this type of hemolysis is termed β-hemolysis (119). This discussion will concentrate on organic hemolysins, especially toxins, because not only do they lyse erythrocytes, but they are also potent cytolysins (13). Most cytolytic toxins have certain structural and functional properties in common, including the possession of a terminal sulfhydryl (SH)-group which binds with sterol groups within the cell membrane of mammalian cells and initiates the lysis of these cells (13, 57). The mechanisms by which these cytolysins lyse cells are extensively explained (13).

After examining one hundred *Listeria* strains, Ralovich *et al.* (130) noted that non-hemolytic *Listeria* strains never changed their hemolytic characteristics and that β-hemolysis was linked to virulence in each of the *Listeria* strains that were examined. This was contradictory to previous findings (114, 115). Moreover, he concluded that the hemolytic property of *Listeria* spp. was genetically stable and that changes in the media constituents may obscure the noticeability of β-hemolysis (130).

Recent evidence suggests that β-hemolysis by pathogenic *Listeria spp.* is caused by the LLO toxin and that this toxin correlates with virulence (14, 28, 24, 52, 53, 82, 125). The LLO toxin is a cytolytic toxin produced by *L.monocytogenes, L.ivanovii* and *L.seeligeri* (61, 95) and is grouped together with other cytolytic toxins of other Gram-positive bacteria, because all cytolytic toxins are structurally and functionally similar (13).

To date, only two *L.monocytogenes* strains were found to be hemolytic but avirulent (130). These are *Listeria* strain NCTC 10357 25/4/4 which was avirulent in mouse and Anton's pathogenicity tests and *Listeria* strain NCTC 5105 which was avirulent in the Anton's pathogenicity test only (130). Hof (69) speculates that these strains could be mutant *Listeria* strains because they form part of the Murray Strain Collection, which was collected in 1929 (113) and might have "mutated" during the 58 years of storage.

1.5.5 The nature of the LLO toxin among different *Listeria* species

The *Listeria* species *L.innocua, L.welshimeri, L.grayi, L.murrayi* and *L.denitrificans*, do not produce LLO toxin, are non-hemolytic and are avirulent (56, 61, 95, 173).

Although the evidence suggests that the LLO toxin produced by *L.monocytogenes* plays a major role in virulence, uncertainty remains about the hemolysin produced by *L.ivanovii* and *L.seeligeri* (87, 95, 165). All *L.ivanovii* strains are highly β-hemolytic and produce ten times more LLO toxin than *L.monocytogenes* and are highly pathogenic to animals, but not to humans (43). *L.seeligeri* strains are weakly hemolytic and produce a tenth of the LLO toxin produced by *L.monocytogenes* (43) and were linked to only one human infection (95). If the LLO toxin plays a major role in virulence, then it is expected that *L.ivanovii* should be highly virulent in comparison to *L.monocytogenes,* because it produces greater amounts of the LLO toxin, but this is not the case.

Experiments were done to investigate the difference between the LLO toxin produced by *L.monocytogenes, L.seeligeri* and *L.ivanovii.* In DNA hybridization studies, using five gene probes consisting of the gene that codes for the LLO toxin and surrounding LLO gene regions, highly homologous LLO genes were detected in species of *L.monocytogenes, L.seeligeri* and *L.ivanovii* and immunoblotting performed with affinity purified anti-LLO antibodies, detected the LLO toxin in all three species (95). This study indicated that the LLO toxins of all three *Listeria* species are antigenically similar (95). It was also found that the restriction maps of the LLO gene region were homologous in all three species, except for a flanking region, 1400 bp downstream of the LLO gene (95). The downstream region differed in all three species and this flanking region was postulated to be the control region for the expression of the LLO toxin (95). Furthermore, comparative studies of the N-terminal sequence of the LLO toxin from *L.monocytogenes* and *L.ivanovii* revealed that the amino acid sequence was highly conserved, 11 out of the 22 amino acids were identical, including the first three amino acids (87). Whether these differences in certain amino acids, including differences in the postulated

control region, are responsible for the differences in the degree of virulence, is still unclear.

1.5.6 The specific role of the LLO toxin in virulence

Autopsies of listeriosis victims indicated that the cause of death in humans was generally due to either meningitis and/or pneumonia and/or respiratory and/or heart failure (23, 54, 151). The appearances of milliary granulomas (masses of inflamed tissues made up of many small lesions), focal necrosis and/or suppuration (pus formation) are common histopathological characteristics of human organs (lungs, liver, spleen, meninx and the lymphatic structure of the small intestine) of individuals suffering from listeriosis (105, 143). Similar histopathological changes were observed in experimental animals artificially infected with *L.monocytogenes* (85, 86, 105). Electron microscopic studies of affected animal spleen tissue indicated that the membranes of these cells were disrupted (85). It was suggested that a lytic factor was responsible for the disruption of the membranes (85, 86). Since a "Hemolysin" was previously identified in *L.monocytogenes* (76) it was postulated that this hemolysin was responsible for the observed effects (85).

An experiment was constructed to examine whether purified hemolysin had the same postulated effect as suggested (85). Liver and peritoneal cells (cells that contain macrophages and large granule fraction cells (LGF)) were isolated from rabbits and rats (85, 86). These were, *in vitro,* infected with purified hemolysin to see whether lysis of lysosomes by hemolysin caused the release of specific lysosome-associated enzymes (85). A direct correlation was found between the amount of lysosome-associated enzymes released and the dosage of hemolysin used (85). Furthermore, no increase in the lysosome-associated enzymes were noted in the plasma of mice which were previously immunized with small dosages of hemolysin and re-exposed to toxic levels of the same

hemolysin (85). Another study, conducted on mice, indicated a direct relationship between cardiotoxicity (variation in the cardiograph readings), death and the dosage of purified hemolysin that was used (86). Furthermore, a dosage of purified LLO toxin of 0.8 μg/10-20 g mouse body weight was lethal to mice (57).

The β-hemolysis factor was isolated and fully characterized, for the first time, in 1976 (57) and was termed Listeriolysin O (LLO) toxin. A subsequent experiment proved that the LLO toxin secreted by *L.monocytogenes* was the only protein that bound to erythrocytes during the hemolytic process (82). Furthermore, site directed mutations in the LLO gene of virulent, β-hemolytic strains of *L.monocytogenes*, resulted in mutant (hly^-) strains that were unable to produce a 58 kDa LLO toxin, lacked the ability to lyse red blood cells and were non-virulent in mouse virulence tests (53, 82), whereas spontaneous loss of the transposons resulted in the restoration of the LLO toxin production and virulence. Moreover, these hly^- *L.monocytogenes* mutants were rapidly eliminated from the spleen (24 hours) and liver (48 hours) of mice which were artificially infected with these mutants (53, 82, 125), whilst the wild type continued to grow in these tissues.

Also, only LLO producing *L.monocytogenes* strains survived and multiplied within mouse peritoneal macrophages whereas, mutant strains lacking the hly gene showed reduced survival within these macrophages (89), but both the wild type as well as the mutant strains were equally well taken up by the macrophages through an endocytosis process (89), thus proving that the LLO toxin was not essential for the uptake of *L.monocytogenes* by the macrophages.

Moreover, protective immunity against virulent wild type *L.monocytogenes* was induced by immunisation with LLO producing, rough mutants of

L.monocytogenes which did not multiply inside the host cells (52, 53). Another experiment proved that LLO producing *L.monocytogenes* strains prevented macrophages from antigen processing and presentation (a common process by which macrophages induce antibody and cellular immune responses that assist in the destruction of the antigen) (26). Avirulent strains did not prevent antigen presentation and processing (26).

The possibility existed that insertional inactivation of hly A gene with transposons (26, 52, 53) might have had a polar effect on other genes adjacent to the hly A gene. These other genes could also be involved in the expression of other virulence factors that are required for entry, intracellular survival and multiplication of *L.monocytogenes* within host tissues. Hence, mutation of the hly A gene by transposons could also affect adjacent genes and therefore, observation of a change in virulence of *L.monocytogenes* does not conclusively indicate that mutation of the hly A gene was solely responsible for the observed virulence changes. An experiment was done to show that the hly A gene was solely responsible for virulence of *L.monocytogenes*. Using a hly⁻ *L.monocytogenes* mutant and gene complementation technique, Cossart *et al.* (28) transformed this mutant with a plasmid carrying a hly A gene. This transformed bacterium was hemolytic and virulent, thus excluding the possibility of polar effects and clearly showed that a single gene the hly A gene was responsible for virulence of *L.monocytogenes* (28).

Finally, the hly A gene was cloned into a non-virulent, common soil bacterium *Bacillus subtilis* and the transformed bacterium was hemolytic and lysed the phagosome membranes of mammalian cell line J774 (14). In addition this mutant bacterium grew rapidly within the cytoplasm of these cells. These results conclusively illustrated that a single gene product, the LLO toxin was responsible for the survival, multiplication and escape of *B.subtilis* from

phagosomes (14) and possibly the same is true for virulent *L.monocytogenes* strains.

The mechanism by which the LLO toxin ensures the survival and multiplication of *L.monocytogenes* within the spleen and liver phagocytes is not fully understood, but extrapolating from above studies and observations it was (89, 125) speculated that once *L.monocytogenes* is taken up by the spleen and liver phagosomes (the LLO toxin possibility not involved in this step) the bacterium secrete the LLO toxin which lyse the phagosome membrane and allows the bacterium to escape into the cytoplasm of the phagocytes where the bacterium is protected from the bactericidal action of lysosome-associated enzymes (89). Moreover, it has been speculated that the necrosis and suppuration seen on the spleen and liver of listeriosis victims is due to secondary effects caused by degranulation and the build-up of lysosomal enzymes and leucocytes, especially polymorphonuclear granulocytes, in the affected tissues (81, 82, 105).

Therefore, β-hemolysis occurs by the action of the LLO toxin and this toxin is a major virulence factor that determines the survival and multiplication of *L.monocytogenes* within spleen and liver phagocytes. The LLO toxin lyse the phagosomes and thus plays a role in the escape of this bacterium from phagosomes and the bacterium is thus protected from destruction by lysosome enzymes. Also, the LLO toxin prevents antigen processing by phagocytes and thus prevent T-cell recognition by a mechanism which is still not clear.

1.5.7 Purification of the LLO toxin and characterization of the LLO gene

Several attempts have been made to isolate and characterize the hemolysin produced by *Listeria.* In 1963, Njoku-obi *et al.* (114), proved that

the supernatant fluid (SNF), obtained after centrifugal removal of *L.monocytogenes* from brain heart infusion media in which the bacterium was cultured, was hemolytic. Treatment of the SNF with proteolytic enzymes resulted in SNF that was non-hemolytic, thus suggesting that the hemolysin was a protein moiety (114). Furthermore, ammonium sulphate concentration of the SNF yielded a factor which had increased hemolytic activity. Since a hemolytic exotoxin was isolated from *Streptococcus*, termed Streptolysin O (SLO) in 1941, it was speculated that this hemolytic factor produced by *L.monocytogenes* was similar to the SLO toxin, because both bacteria were Gram-positive and the factor was hemolytic and secreted into the media (75, 76).

In 1971, using the same technique used for the isolation of SLO, Jenkins and Watson (75) purified a 170 kDa hemolytic protein from a virulent *L.monocytogenes* strain 7973. Similar tests, as previously done with purified SLO, were done on this purified factor (75). The hemolytic activity of this protein was inhibited by various oxidizing agents and increased by reducing agents like cysteine (75). This protein cross-reacted with anti-SLO antibodies and was lethal to mice (75). All of these results confirmed speculations that the hemolysin produced by *L.monocytogenes* was an endotoxin, similar in structure and function to SLO toxin (55-60 kDa), except it was larger and also exhibited lipolytic activity (75).

In 1987, Geoffroy *et al.* (57), using thiol disulphide affinity chromatography, isolated and fully characterized the hemolysin. This hemolysin was isolated from *L.monocytogenes* strain EGD serovar 1/2a and was termed α-Listeriolysin O (LLO) (57) in accordance with nomenclature guidelines recommended by Bernhimer and Rudy (13) and because this toxin was similar to other cytolysin toxins (Table 3). This LLO toxin had similar properties to the hemolysin that was previously isolated (75), but the size of this LLO toxin was about 60 kDa

and no lipolytic activity was found (57). The size (60 kDa) and properties such as hemolysis, no lipolytic activity, cross-reaction with anti-SLO antibodies, hemolysis inhibition by oxidizing agents and activation by reducing agents such as cysteine were similar for the LLO toxin produced by different *L.monocytogenes* strains and this was confirmed by other studies (87, 110, 165).

Subsequent to the isolation and identification of the LLO toxin (57) it was found that only hemolytic, virulent *Listeria* species produced a similar LLO toxin, but Parrius *et al.* (117) found that crude LLO toxin, of 26 out of 28 hemolytic, virulent *L.monocytogenes* strains, could not cross-react with anti-α-LLO antibodies, yet these 26 strains produced a 60 kDa extracellular protein (as seen from the presence of a 60 kDa band on the sodium dodecyl sulphate polyacrylamide gels). They speculated that there were two immunologically distinct LLO toxins produced by virulent, hemolytic *L.monocytogenes* strains and proposed that the hemolysin produced by these 26 strains should be termed β-Listeriolysin O (117).

In 1988, Mengaud *et al.* (110) sequenced the entire hemolysin gene (hly A) of *L.monocytogenes* that coded for α-Listeriolysin O. The hly A gene was cloned into *Escherichia coli*. Expression of this gene occurred and the hemolysin (about 58 kDa) produced by the transformed *E.coli* was detected with specific anti-LLO antibodies illustrating that this hemolysin was antigenically similar to the LLO toxin (110). Furthermore, the nucleotides (504 base pairs) of the hly A gene corresponded with the amino acid sequence of the purified Listeriolysin O toxin and the nucleotide sequence of the hly A gene was highly homologous to the nucleotide sequence of genes that codes for Streptolysin O and Pneumolysin toxins (110). The latter toxins are related to the LLO toxin (13).

In 1992, Michel and Cossart (111) mapped the entire chromosome of *L.monocytogenes* and found that a single hly A gene was present on the chromosome and this gene formed part of a gene cluster. The other genes in this gene cluster coded for other factors which were also implicated in virulence of *L.monocytogenes* (79, 109, 124).

Therefore, it appears that *L.monocytogenes* strains produce only one type of Listeriolysin O toxin (α-LLO) and that this toxin is coded for by a single gene (hly A).

1.5.8 LLO toxin produced by different *Listeria* species

Although the LLO toxin was isolated from *L.monocytogenes* EDG serovar 1/2a (57), a 58 kDa LLO toxin was subsequently purified from the SNF of *L.monocytogenes* Sv4b and from *L.ivanovii* American Type Culture Collection (ATCC) 19119 (87). In 1989, a 61 kDa LLO toxin was isolated from the same *L.ivanovii* ATCC 19119 strain (165). The hly A gene was also detected, for the first time, in *L.seeligeri* using a gene probe specific for the LLO gene (95). Prior to the detection of the hly A gene there was uncertainty about the nature of the hemolysin produced by *L.seeligeri*, because this *Listeria* species produces β-hemolysis which is not easily noticeable on sheep blood agar and was not detectable with anti-LLO antibodies (61), but subsequently, a 60 kDa LLO toxin was detected in the SNF of *L.seeligeri* using affinity purified LLO antisera (56). The LLO toxin was never isolated or detected from avirulent *Listeria* species (56, 95) further suggesting that LLO toxin is a unique factor involved in the pathogenicity of virulent *Listeria* species. The LLO toxin produced by *L.ivanovii* and *L.seeligeri* were termed respectively, Ivanolysin O and Seeligeriolysin O, to indicate the *Listeria* species from which they were isolated (Table 3).

TABLE 3. Classification of SH-activated cytolysins[a]

Genus	Species	Toxin
Streptococcus	*S.pyogenes*	Streptolysins O
	S.pneumoniae	Pneumolysin
Bacillus	*B.cereus*	Cerolysin
	B.thuringiensis	Thuringiolysin O
	B.alvei	Alveolysin
	B.laterosporus	Laterosporolysin
Clostridium	*C.bifermentans*	Bifermentolysin
	C.botulinum	Botulinolysin
	C.histolyticum	Histolyticolysin
	C.novyi type A	Oedematolysin O
	C.perfringens	Perfringolysin O
	C.septicum	Septicolysin
	C.tetani	Tetanolysin
	C.chauvoei	Chauveolysin
Listeria	*L.monocytogenes*	Listeriolysin O
	L.ivanovii	Ivanolysin O
	L.seeligeri	Seeligeriolysin O

[a]From Bernhimer and Rudy (13) with modification.

1.5.9 Biochemical and physical properties of the LLO toxin

The LLO toxin is structurally and functionally similar to other cytolytic toxins which vary in size from 48-68 kDa and all are antigenically related to one another (13). All these toxins are produced by Gram-positive bacteria and are exotoxins, except for Pneumolysin O which is an endotoxin (13). All these cytolytic toxins have an essential sulfhydryl (SH) group which binds to sterol sites within the host's cell membranes, resulting in the lysis of these membranes (13). The cytolytic activity is inhibited by mild oxidation, cholesterol as well as closely related sterols (13). These toxins are reactivated

by reducing agents such as cysteine. Oxidation agents such as sterols and reducing agents bind covalently to the SH-group of the toxin and hence the term SH-activated toxin (13).

The LLO toxin differs from other cytolytic toxins since it is more heat-resistant (lethality for mice was lost at 60°C, 1 hour in phosphate buffer saline pH 6.8) and its lethality is fully active at pH 5.5, but its lethality for mice was lost at pH 7.0 (57), whereas the other toxins are fully active at pH ≥ 6.8 to pH 7.0 (13).

The ability for *L.monocytogenes* to survive within and cause damage to macrophages and phagocytes, which have an acidic internal environment, is possibly due to the LLO toxin which can withstand low pH (158). Therefore, it was speculated that the LLO toxin can cause more damage to its host than any of the other closely related cytolysins (158).

1.5.10 Virulence tests for the identification of pathogenic *Listeria* species

Although, *β*-hemolysis on blood agar has been used since 1932 to differentiate between virulent and non-virulent *Listeria* species, nonetheless there are disadvantages associated with this test (129, 139, 155). It has been observed that *Listeria* species produce hemolysis of variable intensities (strong, weak to unobservable) and that the ingredients within the media used for blood overlays (37, 130), the type of blood (human, sheep, horse) (132) can affect hemolysis. Furthermore, it has been observed that *L.monocytogenes* produces low amounts of hemolysin, whereas *L.seeligeri* produces high amounts at pH 9 at 20°C and that various temperatures also have an effect on the amount of hemolysin produced by different *Listeria* species (149). These factors make the usage of hemolysis as an indicator of virulence unreliable. Hence it was suggested that hemolysis *per se* is not a

good marker to use, to differentiate between virulent and avirulent *Listeria* strains, but that the presence of certain hemolysin(s) such as Listeriolysin O should be ascertained to determine the pathogenicity of *Listeria* strains (129, 155).

Alternative tests based on the usage of animals were introduced. In the Anton's test, the conjunctival sac of the eyes of live animals such as rabbits, guinea pigs or monkeys are injected with *Listeria* and the development of conjunctivitis within 4-14 days indicated that the bacterium was virulent (69, 130). The mouse pathogenicity test involved the injection of mice with 10^3 - 10^9 *Listeria* cells/ml and the *Listeria* strain with the lowest LD_{50} that caused death of mice within 6-21 days was regard as virulent (69, 130). In the chick embryo virulence test, the yolk or allantoic membrane of 11-17 old embryos are injected with *Listeria*, death of the embryos within 3-14 days indicated a virulent bacterium (139, 159). Virulence tests using animals are costly and time consuming and hence more rapid and cost-saving tests, alternative to the animal tests were introduced.

One of the tests that were introduced involved a single CAMP (25) test specific for *Listeria* (63). In this test *Staphylococcus aureus* was streaked vertically on sheep or horse blood agar plates and *Listeria* strains were streaked horizontally at right angles to the *S.aureus* streak so as not to touch *S.aureus* (about 1 cm away) (63). Observation of enhanced synergism (indicated by an arrow-head hemolytic pattern) of *Listeria* in the region of *S.aureus* after incubation at 37°C for 24-48 days indicated that this *Listeria* strain was virulent (63). A different modification of the CAMP test was the use of partially purified beta toxin of *S.aureus* and equi-factor of *Rhodococcus equi* in the double CAMP test (150, 154). These strains or their exosubstances were streaked vertically and parallel to each other and *Listeria* strains were streaked horizontally at right angles between these

strains. Enhanced synergism of *Listeria* with both or either of the strains indicated a virulent *Listeria* strain (150, 154). Groves *et al.* (63) showed a correlation between the CAMP test, fermentation of L-rhamnose and non-fermentation of D-xylose (CRX) and mouse pathogenicity tests of virulent *Listeria* species and suggested that the CRX test could be used as a routine, rapid and cost saving test to differentiate between pathogenic and non-pathogenic *Listeria* species. According to the CRX test virulent *Listeria* species were positive in the CAMP test, fermented L-rhamnose and did not ferment D-xylose (63).

More recently natural gene probes (33, 34) and a synthetic oligonucleotide of 20 base pairs (35), based on the sequence of the hly A gene have been used to detect virulent *Listeria* species. DNA hybridization using these probes are rapid in identifying virulent *Listeria* species, but these probes still needs to be tested more extensively in order to establish the reliability of these probes to specifically detect virulent *Listeria* species.

1.6 OBJECTIVES OF THIS STUDY

In Europe and America high incidence of human mortality occurred which was associated with foodborne listeriosis. The etiological agent of listeriosis was identified as *Listeria monocytogenes* (43). Furthermore, in America and Europe, *Listeria* is reported to be ubiquitous in nature, occurring as part of the natural flora of soil, water, and various animals (16). Also, sufficient evidence indicates that the pathogenesis of international isolates of *Listeria* is linked to the production of a major virulence factor known as α-LLO toxin (14, 28, 56). By contrast to Europe and America, in South Africa, one outbreak of listeriosis (73) and sporadic cases of listeriosis have been reported (1, 126) and none of these cases were linked to consumption of *Listeria*-contaminated food. Furthermore, in South Africa and more specifically in the Western Cape nothing is known about the virulence nature of *Listeria* found in raw milk and raw chicken. Therefore, the objective of this study was to assess whether 12 *Listeria* isolates from raw milk (4 isolates) and raw chicken (8 isolates) in the Western Cape, South Africa, were pathogenic by the CAMP-rhamnose-xylose test (CRX) and whether this phenotype was linked to the detection of the α-LLO toxin by immunological assays and the detection of the Listeriolysin O gene (hly A) by DNA hybridization using two oligonucleotide probes.

CHAPTER 2

2 ISOLATION AND CHARACTERISATION OF *LISTERIA* FROM RAW MILK AND RAW CHICKEN

2.1 SUMMARY

Listeria **was isolated from raw milk and raw chicken in the Western Cape, South Africa, with the main aim of determining the virulence of these *Listeria* isolates. Two raw milk samples, collected from bulk tanks in the Western Cape, were analyzed for *Listeria* by the United States of America's Department of Agriculture Food Sanitation Inspective Services (FSIS) and the United States of America's Food and Drug Administration (FDA) methods. Four *Listeria* isolates (DB121, SK118, SK133 and SK135) were isolated from both raw milk samples. Isolates DB121 and SK135 were identified as *Listeria monocytogenes* by morphological and biochemical tests as well as by the *Listeria* API identification system and isolate SK133 was identified as *L.grayi*, whereas isolate SK118 could only be identified as *L.innocua* using the *Listeria* API identification system and not by morphological and biochemical tests. Eighteen raw chicken samples, taken from one poultry processing plant in the Western Cape, were analyzed by a modified FSIS method and eight isolates (isolated from 8 samples) were identified as *L.monocytogenes* using the *Listeria* API identification system**

2.2 INTRODUCTION

High incidence of human mortality associated with foodborne listeriosis has been reported in America and Europe (43) and the etiological agent *Listeria monocytogenes* was isolated from various types of food (40).

Most of the listeriosis outbreaks were associated with the consumption of dairy products and high incidence of *Listeria* was found in raw milk (97, 104, 134). *Listeria* was consistently found in raw chicken (77, 90, 121), but chicken has never been implicated in listeriosis outbreaks (43). By contrast to America and Europe, none of the sporadic cases (1, 126) nor the one major outbreak (73) of listeriosis in South Africa were linked to food-associated listeriosis. Furthermore, no information about the virulence nature of *Listeria* present in raw milk and raw chicken in the Western Cape, South Africa was found.

Several methods have been used for the enrichment and isolation of *Listeria* (41, 66, 93, 100). However, several factors determine the suitability of a specific method, these include the type of food analyzed, purpose of analysis, time and cost. The Food and Drug Administration (FDA) (99) and the Food Services and Inspection Services (FSIS) (93) methods are most successfully used in America and Europe (127, 128). The FDA method, uses a single enrichment procedure and was specifically designed for dairy products (100), whereas the FSIS method was designed for meat and poultry products and uses a double enrichment procedure (93). Both methods are now widely used in the enrichment and isolation of *Listeria* from most foods (127, 128).

Rapid methods such as DNA-hybridization using natural or synthetic probes (33, 34, 35, 84), polymerase chain reaction (36), magnetic immuno-polymerase chain reaction (49), flow cytometry (39), enzyme-linked immunosorbent assay (ELISA) (45) and multilocus enzyme electrophoresis (20) have also been used for the detection of *Listeria* in food, but most of these methods require a FDA or FSIS pre-enrichment of the sample, prior to further analysis. Serology (no comprehensive sera set is commercially available) (144), DNA-DNA and rRNA-DNA hybridization (78, 145) are also

used and are useful when morphological and biochemical tests are inadequate in differentiating between closely related *Listeria* species. Serology is limited to the differentiation of epidemiological important *L.monocytogenes* serovars (144).

Nevertheless, in many reports, isolation of *Listeria* followed by identification using morphology and biochemical tests are still used (41, 66, 93, 100, 127, 128). In 1992, a new *Listeria* API identification system (BioMérieux, La Balme-les-Grottes, France) was released which could identify *Listeria* species within 18-24 hours as opposed to the 7-14 days which is required for morphological and biochemical identification methods.

The objectives of this study were to isolate *Listeria* from raw milk and raw chicken in the Western Cape, South Africa with the main aim of determining the virulence nature of any *Listeria* that was isolated. Two raw milk samples and 18 raw chicken samples were examined by the FDA and the FSIS methods and morphological and biochemical methods as well as the API *Listeria* identification system were used to identify *Listeria* species.

2.3 MATERIALS AND METHODS

2.3.1 Strains and media

Two reference strains were used, a non-hemolytic *Listeria monocytogenes* strain (Irene Animal Production Institute (IAPI)) and a β-hemolytic *L. monocytogenes* (National Collection of Type Cultures (NCTC) 7973 strain (U. Kreft; pers. comm.[3]). The bacteria were maintained and subcultured weekly on trypticase soy agar (OXOID Limited, Basingstoke,

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[3] U. Kreft, University of Würzburg, 8700 Würzburg, Röntgenring 11, Germany.

England) supplemented with 0.6% yeast extract (MERCK, Darmstadt, Germany) (TSA-YE) and stored at 4°C. Subcultures were made from TSA-YE slants and streaked onto TSA-YE plates and incubated at 37°C for 24 hours before being used in experiments.

2.3.2 Sample collection

Two fresh raw milk samples (75 ml each) were aseptically collected into sterile Duran bottles from bulk collection tanks from two different farms (1 sample from each) in the Western Cape, South Africa. The raw chicken samples were collected and analyzed for *Listeria* by Gouws (P. Gouws; pers. comm.[1]) and a brief description of the method used follows: eighteen samples of raw chicken (10 swab samples from the cloacae and 8 swab samples from the neck skins) were, individually and aseptically, collected into sterile Duran bottles containing 50 ml of sterile distilled water (P. Gouws; pers. comm.[1]) from one poultry processing plant in the Western Cape, South Africa. The raw milk and raw chicken samples were chilled by transporting the samples on ice inside a cooler bag. At the laboratory the raw milk and chicken samples were immediately analyzed for *Listeria.*

2.3.3 Enrichment and isolation of *Listeria*

Each of the raw milk samples were examined for *Listeria* by FDA (99) and FSIS (93) methods, simultaneously. The FSIS method (93) involved the aseptic transfer of 25 ml raw milk sample into 225 ml pre-enrichment broth, *Listeria* Enriched Broth Base, University of Vermont (UVM) formulation (OXOID Limited, Basingstoke, England) with supplement SR 142 (OXOID Limited, Basingstoke, England). The inoculated broth were

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[1] P. Gouws, University of the Western Cape, Private Bag X17, 7535 Bellville, South Africa.

incubated at 28°C for 24 hours. After incubation, 25 ml pre-enriched inoculum was aseptically transferred into 225 ml secondary enrichment broth, *Listeria* Enrichment Broth Base (UVM formulation) (OXOID Limited, Basingstoke, England) with supplement SR 143 (OXOID Limited, Basingstoke, England) and incubated at 28°C for 72 hours. Duplicate 10-fold serial dilutions of the secondary enriched raw milk samples were aseptically made in 0.5% potassium hydroxide (KOH) (Sigma Chemical Company, St. Louis, USA) dilution fluid as recommended (99) and 0.1 ml of each dilution and 0.1 ml of undiluted secondary enriched inoculum were aseptically spread plated onto duplicate plates of *Listeria* Selective Agar Base (OXOID Limited, Basingstoke, England), McBride Agar (OXOID Limited, Basingstoke, England) and Modified McBride Agar (OXOID Limited, Basingstoke, England), respectively. Plates were incubated at 37°C for 24 hours.

The FDA method as explained by Lovett (99) was followed and involved the aseptic inoculation of 25 ml of raw milk sample into 225 ml *Listeria* Enrichment Broth Base (OXOID Limited, Basingstoke, England) supplement with SR 141 (OXOID Limited, Basingstoke, England). The inoculated *Listeria* Enrichment Broth Base (OXOID Limited, Basingstoke, England) was incubated at 30°C for 2, 4 and 7 days. At 2, 4 and 7 days, duplicate 10-fold serial dilutions were made in 0.5% KOH (Sigma Chemical Company, St. Louis, USA) dilution fluid as recommended (99) and 0.1 ml of each dilution and 0.1 ml of undiluted enriched inoculum, were spread plated onto duplicate plates of *Listeria* Selective Agar Base (OXOID Limited, Basingstoke, England), McBride Agar (OXOID Limited, Basingstoke, England) and Modified McBride Agar (OXOID Limited, Basingstoke, England), respectively. Plates were incubated at 37°C for 24 hours.

The raw chicken samples were enriched and analyzed for *Listeria* according to the FSIS method as modified by Bridson (21)(P. Gouws; pers. comm.[1]) . The method involved aseptic inoculation of 25 ml raw chicken samples into 225 ml of *Listeria* Primary Selective Enrichment media (UVM 1) (OXOID Limited, Basingstoke, England) and the inoculated media were incubated at 30°C for 24 hours. After incubation 0.1 ml primary enriched sample was aseptically transferred into 9.9 ml of *Listeria* Secondary Selective Enrichment media (UVM 2) (OXOID Limited, Basingstoke, England) and incubated at 30°C for 24 hours. Both the primary and secondary enriched raw chicken samples (1 ml) were separately and aseptically diluted in 9 ml of 0.5% KOH (Sigma Chemical Company, St. Louis, USA) dilution solution. Undiluted and diluted primary and secondary enriched chicken samples (0.1 ml) were aseptically spread plated on *Listeria* Selective Agar (OXOID Limited, Basingstoke, England) and incubated at 30°C for 24 hours.

2.3.4 Selection and identification of *Listeria*

After incubated at 35°C for 24 hours undiluted and diluted (10^{-1}-10^{-9}) FDA and FSIS enriched raw milk samples on Modified McBride Agar, McBride Agar and *Listeria* Selective Agar (OXOID Limited, Basingstoke, England), were examined for *Listeria* as recommended (99). All isolated colonies that were blueish in colour on McBride Agar and Modified McBride Agar, (when viewed under a stereo-microscope with a light source illuminating the plates at an incidence angle of 45°C) (99) and colonies that were small dark green (surrounded by black zones) on *Listeria* Selective Agar (31), were selected and following the scheme recommended by Lovett (99), selected colonies were picked and individually streaked onto TSA-YE plates and plates were incubated at 37°C for 24 hours. After incubation,

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[1] P. Gouws, University of the Western Cape, Private Bag X17, 7535 Bellville, South Africa.

isolated colonies were picked from TSA-YE plates and streaked onto fresh TSA-YE media and this inoculated media was incubated at 25°C for 24 hours in order to determine the motility of the reference strain and the isolates as suggested by Lovett (99) using the wet mount hanging drop method (64). Isolates that exhibited typical slow-rock-tumble motilities (99) were picked from TSA-YE plates and streaked onto fresh TSA-YE media and incubated at 37°C for 24 hours. After incubation these isolates and the reference strain were subjected to Gram stain, catalase and oxidase tests as recommended by Lovett (99). Further identification of presumptive *Listeria* that were Gram positive, catalase positive, oxidase negative and which exhibited slow-rock-tumble motility was done by determining biochemical phenotypes as defined in Bergey's manual (145) and also by using the *Listeria* API identification system (BioMérieux, La Balme-les-Grottes, France). The same selection scheme as explained above was used for the analysis of raw chicken samples for *Listeria*, except that only the *Listeria* API identification system was used for the identification of presumptive *Listeria* isolates (P. Gouws; pers. comm.[1])

2.3.5 Biochemical tests and *Listeria* API identification system

All biochemical tests as defined in Bergey's manual (145) were done using standard methods (64) and consisted of the following tests: oxidase, catalase, methyl red, Voges Proskauer (Barrit's modification (64)); fermentation of: D-glucose, L-arabinose, mannitol, melibiose, L-rhamnose, D-xylose, maltose, salicin (peptone water basal medium with 0.002 % bromocresol purple indicator pH 7 was used in each of the carbohydrate tests); hydrolysis of: cellulose, casein (powder milk), gelatin (Frazer's modified gelatin agar), starch; reduction of NO_3 to NO_2 (modified Griess-

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Ilosvay's and mercuric chloride reagents); production of indole from tryptophan (Kovak's reagent); ammonia production from urea (Christensen's urea agar); utilization of citrate as sole carbon source (Simmon's citrate agar) and β-hemolysis (Nutrient Agar containing 5% sheep blood) (64).

The *Listeria* API identification system (BioMérieux, La Balme-les-Grottes, France) reaction strip consists of the following tests: absence or presence of arylamidase (DIM); esculin hydrolysis (ESC); absence or presence of α-manosidase (α-MAN); acidification of: D-arabitol (DARL), D-xylose (XYL), rhamnose (RHA), α-methyl-D-Glucoside (MDG), ribose (RIB), Glucose-1-Phosphate (G1P) and D-tagatose (TAG).

The *Listeria* API identification system was used according to manufactures instructions. Presumptive *Listeria* isolates which were grown for 24 hours at 37°C were aseptically removed from trypticase soy agar (OXOID) plates with sterile toothpicks and resuspended in 2 ml of sterile distilled water (supplied by the manufacturer in a glass vial) to an opacity of 0.5 Macfarland standard. The incubation tray (supplied by manufacturer) was filled with 5 ml of sterile distilled water to create a moist atmosphere and the reaction strip was removed from its packaging and placed in the incubation tray. The bacterial suspension was then aseptically pipetted into the 10 microtubes (100 μl for the DIM test and 50 μl for the other tests). The incubation tray was incubated at 37°C for 24 hours. After incubation one drop of ZYM B (supplied by the manufacturer) was added to the DIM test and allowed to react for 3 minutes and then all reactions were read according to the colour changes as indicated in the manufacturer's instructions. The *Listeria* profiles were determined according to the manufacturer's instructions.

2.4 RESULTS

A total of 48 colonies were selected from both raw milk samples as follows: 28 colonies on McBride Agar and 12 colonies on Modified McBride Agar that were bluish under transillumination microscopy and 8 small dark green colonies, surrounded by black zones (esculin hydrolysis) on *Listeria* Selective Agar (Table 1). Of these 48 colonies 27 colonies were selected after enrichment by the FSIS method, whereas 21 colonies were selected after the FDA enrichment method. Only 4 out of 48 isolates from raw milk were Gram positive rods, motile at 25°C (slow rock tumble motility), catalase positive and oxidase negative (Table 2). These four isolates DB121, SK118, SK133, SK135 were identified as *Listeria* species by morphological and biochemical tests (Table 2); by the *Listeria* API identification system (Table 3). The 8 isolates from raw chicken which were Gram positive rods, motile at 25°C (slow rock tumble motility), catalase positive and oxidase negative were identified as *L.monocytogenes* (Table 3) using the *Listeria* API identification system (BioMérieux, La Balme-les-Grottes, France).

2.5 DISCUSSION

Isolate SK118 from raw milk was selected from Modified McBride Agar after FDA enrichment (Table 1). The morphological and biochemical phenotypes (Table 2) of isolate SK118 correlated with the morphological and biochemical phenotypes of *Listeria* (Table 2), except isolate SK118 hydrolyzed casein, whereas none of the known *Listeria* species listed in Bergey's Manual (145) exhibit this characteristic. Initially, it was thought that fermentable sugars present within the TSA-YE media were transferred with isolate SK118 to the casein media, and this might have, as previously

TABLE 1. **Total number of isolates from two raw milk samples selected from Modified McBride Agar and McBride Agar based on transillumination microscopy and from *Listeria* Selective Agar based on esculin hydrolysis, after incubation at 30°C for 24 hours and after enrichment by the Food and Drug Administration (FDA) and the Food Sanitation Inspection Services (FSIS) methods**

Isolation media	Food Sanitation Inspection Services method		Food and Drug Administration method	
	Number of isolates selected	Number of isolates identified as *Listeria*	Number of isolates selected	Number of isolates identified as *Listeria*
Modified McBride Agar	10	0	18	1
McBride Agar	12	0	no growth	0
Listeria Selective Agar	5	3	3	0
Total percentage positive *Listeria*		11.1%		0.048%

TABLE 2. **Morphological and biochemical characteristics of four isolates from raw milk as compared to *L.monocytogenes* reference strains**

Tests done	BM[a]	RS[b]	SK 118	DB 121	SK 133	SK 135
Morphology						
cell shape	r/c	r	r	r	r	r
Gram stain	+	+	+	+	+	+
motility	+	+	+	+	+	+
Biochemical tests						
catalase	+	+	+	+	+	+
oxidase	-	-	-	-	-	-
Methyl red	+	+	+	+	+	+
Voges Proskauer	+	+	+	+	+	+
indole	-	-	-	-	-	-
ammonia	-	-	-	-	-	-
nitrate reduction	-	-	-	-	-	-
Hydrolysis of:						
casein	-	-	+	-	-	-
gelatin	-	-	-	-	-	-
starch	d	-	-	-	-	-
citrate[c]	-	-	-	-	-	-
β-hemolysis	d	-	-	+	-	+
Acid produced:						
D-glucose	d	+	+	+	+	+
L-rhamnose	+	+	-	+	-	+
arabinose	-	-	-	-	-	-
D-mannitol	-	-	+	-	-	-
melibiose	-	-	+	-	-	-
cellulose	-	-	+	-	-	-
maltose	d	+	+	+	+	+
D-xylose	-	-	-	-	-	-
salicin	+	+	+	+	+	+

[a]BM, *L.monocytogenes* as listed in Bergey's Manual (145). [b]RS, reference strain of *L.monocytogenes* (Irene Animal Production Institute (IAPI) or National Collection of Type Cultures (NCTC) 7973, except that *L.monocytogenes* NCTC 7973 was β-hemolytic. [c]Citrate, utilisation of citrate as a sole source of carbon. r, rod; c, coccoid. d, variable (some negative and some positive).

TABLE 3. The phenotypes of four isolates from raw milk and eight isolates from raw chicken as determined by the *Listeria* API identification system

Listeria species	API Tests										β-hem	Code	Species identified
	DIM	ESC	α-MAN	DARYL	XYL	RHA	MDG	RIB	G1P	TAG			
L.monocytogenes	-	+	+	+	-	+	+	-	-	-	+	6510	*L.monocytogenes*
L.ivanovii	-	+	-	-	+	-	+	-	+	-	+	2250	*L.ivanovii*
L.welshimeri	+	+	+	+	+	-	+	-	-	+	-	7311	*L.welshimeri*
DB121	-	+	+	+	-	+	+	-	-	-	+	6510	*L.monocytogenes*
SK118	+	+	+	+	-	-	+	-	-	-	-	7110	*L.innocua*
SK133	+	+	-	+	-	-	-	+	-	-	-	3120	*L.grayi*
SK135	-	+	+	+	-	+	+	-	-	-	+	6510	*L.monocytogenes*
AL1	-	+	+	-	-	-	+	-	-	-	-	6010	*L.monocytogenes*
AL2	-	+	+	-	-	-	+	-	-	-	-	6010	*L.monocytogenes*
AL3	-	+	+	-	-	-	+	-	-	-	-	6010	*L.monocytogenes*
AL5	-	+	+	-	-	-	+	-	-	-	-	6010	*L.monocytogenes*
L31	-	+	+	+	-	+	+	-	-	-	+	6510	*L.monocytogenes*
L35	-	+	+	+	-	+	+	-	-	-	+	6510	*L.monocytogenes*
P73	-	+	+	+	-	+	+	-	-	-	+	6510	*L.monocytogenes*
P75	-	+	+	+	-	+	+	-	-	-	+	6510	*L.monocytogenes*

DIM, arylamidase; ESC, esculin hydrolysis; α-MAN, α-manosidase; DARL, D-arabitol acidification; XYL, D-xylose acidification; RHA, rhamnose acidification; MDG, α-methyl-D-Glucoside acidification; RIB, ribose acidification; G1P, Glucose-1-Phosphate acidification; TAG, D-tagatose; β-hem, β-hemolysis, -, negative reaction; +, positive reaction; code, numerical profiles for positive reactions were determined according to manufacturer's instructions.

suggested (64), resulted in the slight acid production which gave "false positive" results, but subsequent inoculation of isolate SK118 from Nutrient Agar and Nutrient broth (OXOID Limited, Basingstoke, England) confirmed that this bacterium was positive for casein hydrolysis and hence the species affiliation of isolate SK118 could not be determined by morphologial and biochemical tests (Table 2). Nonetheless, using the *Listeria* API identification system, which does not test for casein hydrolysis, isolate SK118 was identified as *L.innocua* (Table 3).

From a total of 27 isolates from raw milk selected from Modified McBride Agar (10 isolates), McBride Agar (12 isolates) and from *Listeria* Selective Agar (5 isolates) after FSIS enrichment, only 3 isolates (Table 1) SK133, SK135 and DB121 were identified as *Listeria* species (Table 2 and 3). From a total of 21 isolates, from the same two raw milk samples, selected from Modified McBride Agar (18 isolates) and 3 isolates selected from *Listeria* Selective Agar after FDA enrichment, only isolate SK118 (Table 1) was identified as *L.innocua* by the *Listeria* API identification system (Table 3). This means that the FSIS two stage enrichment method was more suitable than the FDA one stage enrichment method for the isolation of *Listeria* from these two raw milk samples. Brackett *et al.* (17) also reported that the FSIS enrichment method was a better enrichment method for the isolation of *Listeria* from various foods.

From a total of eight isolates (Table 1) selected from *Listeria* Selective Agar by esculin hydrolysis only three isolates DB121, SK133 and SK135 were identified as *Listeria* species (Table 2 and 3), whereas from a total of 28 isolates selected from Modified McBride Agar, one isolate was identified and from 12 isolates selected from McBride Agar, none were identified as *Listeria*. This means that Listeria Selective Agar was more suitable than the McBride Agar for the isolation of *Listeria* from these two raw milk samples.

Tiwari *et al.* (160) had also found that *Listeria* Selective Agar was more suitable than Modified McBride Agar since *Listeria* Selective Agar inhibited the growth of 50 different types of non-*Listeria* species whereas Modified McBride Agar could not inhibit the growth of these non-*Listeria* species. Although, *Listeria* Selective Agar appears to be more suitable compared to the other two media for the isolation of *Listeria* from these two raw milk samples, 62.5% of the isolates selected by esculin hydrolysis could not be identified as *Listeria*. This suggests that other non-*Listeria* species are also capable of esculin hydrolysis, in fact Al-Zoreky *et al.* (2) found that bacteria such as lactobacilli, staphylococci and enterococci also produced esculin hydrolysis zones on *Listeria* Selective Agar. Furthermore, from a total of 40 isolates selected on Modified McBride (28 isolates) and McBride Agar (12 isolates) by transillumination microscopy (99) only 1 isolate was identified as *Listeria,* whereas 97.5% of the isolates could not be identified as *Listeria*. This implied that transillumination microscopy gave false results and hence it was not a suitable method for selection of presumptive *Listeria* isolates from these two raw milk samples. Tiwari *et al.* (160) also observed that lactobacilli exhibited the same characteristic bluish coloration on Modified McBride agar when viewed under transillumination microscopy.

All *Listeria* colonies that grew on TSA-YE, were white to cream, dome-shaped with a mean colony diameter of about 2 mm. Gram stained cells were short rods to coccoid cells whereas the reference strains of *L.monocytogenes* were distinct, short, slender rods. All *Listeria* isolates from raw milk showed distinct, slow rock-tumble motility at 25°C similar to the slow rock-tumble motility of *L.monocytogenes* IAPI and *L.monocytogenes* NCTC 7973, but both the reference strains and the *Listeria* isolates from raw milk, also exhibited motility after incubation at 37°C for 24 hours on TSA-YE. Observation of motility at the latter temperature is consistent with a report that proved that *Listeria* exhibits

slow motility at 37°C (94), but contradicts reports that claimed that *Listeria* is none motile after incubation at 37°C for 24 hours (145).

2.6 CONCLUSION

L.monocytogenes (DB121 and SK135) was isolated from two raw milk samples collected from bulk tanks in the Western Cape, South Africa, whereas two other *Listeria* species (*L.grayi* and *L.innocua)* were also isolated from only one raw milk sample. The species affiliation of isolate SK118 could not be determined by classical morphology and biochemical test, but according to the *Listeria* API identification system isolate SK118 was identified as *L.innocua* and isolate SK133 was identified as *L.grayi*.

The numbers of presumptive *Listeria* that were isolated from two raw milk after FDA enrichment (27 isolates) compared to the FSIS enrichment (21 isolates) and between *Listeria* Selective Agar (8 isolates), Modified McBride Agar (28 isolates) and McBride Agar (12 isolates) varied and emphasized the importance of choosing the best available method for the isolation of *Listeria* from raw milk. It appears that the FSIS enrichment method and the *Listeria* Selective Agar was best suitable for the isolation of *Listeria* from these two raw milk samples (Table 1).

The incidence of *Listeria* in raw chicken was 44.4% from the 18 samples that were tested and all isolates AL1, AL2, AL3, AL5, L31, L35, P73 and P75 were identified as *L.monocytogenes* (P. Gouws; pers. comm.[1]).

L.monocytogenes (SK135 and DB121) was isolated from two raw milk samples. It is impossible to say whether the incidence of *Listeria* in raw

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milk from bulk collection tanks taken from the Western Cape was significant since only two samples were tested, hence a more comprehensive study should be conducted. Nonetheless, this study resulted in the isolation of four *Listeria* strains from raw milk and eight *L.monocytogenes* from raw chicken. The virulence of these 12 *Listeria* species is described in Chapter 3.

CHAPTER 3

3 DETECTION OF VIRULENCE OF *LISTERIA* ISOLATED FROM RAW MILK AND RAW CHICKEN

3.1 SUMMARY

The virulence of 12 *Listeria* isolates which were isolated from raw milk (4 isolates) and raw chicken (8 isolates) in the Western Cape, South Africa, was determined by the CAMP reaction, rhamnose and xylose acidification (CRX) tests, detection of a 60 kDa *α*-LLO toxin (sodium dodecyl sulphate polyacrylamide gel electrophoresis, Western blotting and immunoblotting) and by oligonucleotide probing to detect the presence of the hly A gene. Six *Listeria* isolates were CRX positive and expressed a 60 kDa protein that gave a positive signal when reacted with anti-*α*-LLO antibodies. Genomic DNA samples of 5 of these 6 isolates also showed strong hybridization signals when reacted with hly A oligonucleotide probes. There was 91.7 % (11 of the 12 isolates) correlation between CRX phenotype, presence of a 60 kDa protein that cross reacted with anti-*α*-LLO antibodies and the detection of the hly A gene by oligonucleotide probing.

3.2 INTRODUCTION

Listeria monocytogenes causes a disease referred to as listeriosis that may be life threatening (54, 105, 151). There has been an alarming international increase in foodborne associated listeriosis mortality since

1981 (45). Furthermore, in America and Europe *Listeria* occurs in various ecological sites such as rivers, lakes, soil, animals, food and humans (16). By contrast, data available on the incidence of listeriosis in South Africa seem to indicate that the problem is almost insignificant. One listeriosis outbreak occurred in Johannesburg in 1978, involving fourteen cases, but no fatalities were reported (73). Sporadic cases of human listeriosis have also been reported (1, 126). None of the listeriosis cases were linked to consumption of *Listeria* contaminated food. Furthermore, 2-5 listeriosis cases are annually recorded in Soweto (H.J. Koornhoff; pers. comm.[1]). Secondly, Wnorowksi *et al.* (175) found that 90.7% of 43 *L.monocytogenes* strains isolated from various dairy products in South Africa contained *L.monocytogenes* serotype 4. *L.monocytogenes* serotype 4 together with serotypes 1/2a, 1/2b and 3b are highly virulent and almost 90% of listeriosis is caused by these strains (54).

Of the seven *Listeria species* only *L.monocytogenes* is a major human pathogen (43). *L.innocua, L.welshimeri, L.grayi* and *L.murrayi* do not produce LLO toxin, are non-hemolytic and non-virulent (107). A LLO toxin was detected and purified from *L.ivanovii* (87, 165) and detected in *L.seeligeri* (56, 95). These two species produce hemolysis of varying intensities and were involved in isolated cases of human listeriosis (43, 61, 95). The reasons for the degree of difference in pathogenicity of these species are still not clear (87, 95).

In 1932, Nyfeldt (116) observed that only *Listeria* species isolated from naturally infected individuals, produced β-hemolysis on sheep blood agar plates and killed mice (virulence test) (116). This led to the assumption that β-hemolysis was correlated with virulence and implied that the factor

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responsible for β-hemolysis was the same factor responsible for pathogenicity (130). Moreover, it implied that observation of β-hemolysis was sufficient evidence that a particular *Listeria* strain was pathogenic. Several earlier studies supported this assumption (71, 130, 131) while other studies contradicted this assumption (58, 114, 115), but more recent evidence, convincingly suggests that β-hemolysis is caused by the LLO toxin and that the LLO toxin correlates with virulence of *Listeria* species (14, 24, 28, 82, 125). Although β-hemolysis is an important virulence marker there are problems associated with using β-hemolysis to differentiate between virulent and non-virulent *Listeria* species. It has been observed that *Listeria* spp. produce hemolysis of variable intensities (37, 130) and that hemolysis is influenced by the type of blood (sheep, human, horse) (132) and the constituents of the nutrient media. For example a non-hemolytic *L.innocua* strain produced hemolysis on blood agar plates containing glucose (37). These factors make the usage of hemolysis as an indicator of virulence unreliable (37). Hence it was suggested that hemolysis *per se* was not a good marker to use, to differentiate between virulent and avirulent *Listeria* strains (129, 155).

Alternative tests based upon usage of animals were also used to identify virulent *Listeria* spp. these include: the Anton's virulence test (69, 130), the mouse pathogenicity test (69, 130) and the chick embryo virulence test (139, 159). These tests normally take 4-21 days to complete (69, 130, 139, 159) and since *Listeria* is a life-threatening pathogen and virulence tests on animals are too costly and time consuming, more efficient and rapid methods are needed to detect and differentiate between virulent and avirulent *Listeria* strains isolated from food, in order to minimize the potential dangers of foodborne transmission of *Listeria* to humans.

Other alternatives to the animal virulence tests, have been used to differentiate between virulent and avirulent *Listeria* species. These include a combination test consisting of a modified classical CAMP (25) and rhamnose -and xylose acidification phenotype (CRX) (63). *Listeria* strains that showed increased hemolytic synergism with *Staphylococcus aureus* (CAMP positive), produced acid from rhamnose and did not acidify xylose were regarded as virulent (63). Skalka *et al.* (150) proved that more reliable CAMP results were obtained if *Rhodococcus equi* or purified *equi*-factor was used instead of *S. aureus* or its β-toxin. It was suggested that the CRX tests may be used as a non-expensive, rapid, routine test for the *in vitro* differentiation of pathogenic and non-pathogenic *Listeria* spp. and especially *L.monocytogenes* strains (63). Another reliable and rapid method of identifying virulent *Listeria* is by DNA-DNA hybridization using synthetic or natural probes (33, 34, 35). The nucleotide sequence of the probes corresponds to the base sequence of the hemolysin (hly A) gene that codes for α-Listeriolysin O toxin (33, 34, 35).

In 1987, Geoffroy *et al.* (57) isolated and fully characterized the α-LLO toxin. It was identified as an exotoxin which is sulfhydryl (SH)-activated, 58-60 kDa in size, hemolytic, cytolytic and antigenically related to other cytolytic toxins (13). Subsequent experiments indicated that it was a major virulence factor which determines the intracellular survival of *Listeria* within host phagocytes (14, 28).

In 1988, Mengaud *et al.* (110) sequenced the entire hly A gene of *L.monocytogenes*. The nucleotide sequence of the hly A gene corresponded with the amino acid sequence of the α-LLO toxin and the nucleotide sequence was highly homologous to the base sequence of genes that coded for Streptolysin O and Pneumolysin toxins. The latter toxins are closely related to α-LLO toxin in terms of structure and function (13).

The aim of this study was to determine whether 12 *Listeria* strains which were isolated from raw milk (4 isolates) and raw chicken (8 isolates) in the Western Cape, South Africa, carried virulence factors. The 12 *Listeria* isolates were examined for the CRX phenotype and presence of a 60 kDa α-LLO toxin by sodium dodecyl sulphate polyacrylamide gel electrophoresis, Western blotting, immunoblotting and by oligonucleotide probing (dot blot) to detect the hly A gene.

3.3 MATERIALS AND METHODS

3.3.1 Strains and media

The following reference strains were used: *Listeria monocytogenes* National Collection of Type Cultures (NCTC) 7973; *Listeria ivanovii* American Type Culture Collection (ATCC) 19119; *Listeria welshimeri* ATCC 5334; *Rhodococcus (Corynebacterium) equi* NCTC 1621; *Staphylococcus aureus* ATCC 25923 and *Listeria* isolates DB121 and SK135 (*L.monocytogenes*); SK133 (*L.grayi*); and SK118 (*L.innocua*) which were isolated from raw milk. Isolates AL1, AL2, AL3, AL5, L31, L35, P73 and P75 (*L.monocytogenes*) were isolated from raw chicken (P. Gouws; pers. comm.[1]). All reference strains and the 12 *Listeria* isolates were maintained on trypticase soy agar (OXOID Limited, Basingstoke, England) slants supplemented with 0.6% yeast extract (MERCK, Darmstadt, Germany) (TSA-YE); except *R.equi* and *S.aureus* which were maintained on Nutrient Agar (MERCK, Darmstadt, Germany) slants, at 4°C. Subcultures were made from the slants onto freshly prepared TSA-YE and incubated at of 37°C for 24 hours before being used in further experiments.

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3.3.2 CAMP test

The CAMP test was performed on sheep blood agar base (OXOID Limited, Basingstoke, England) plates containing 5 % defibrinated sheep blood as previously described (63). *S.aureus* ATCC 25923 and *R.equi* NCTC 1621 were streaked about 4 cm apart, parallel to each other. The test isolates and *L.monocytogenes* NCTC 7973 (positive control), *L.ivanovii* ATCC 19119 (second positive control) and *L.welshimeri* ATCC 5334 (negative control) were streaked in straight lines (± 1 cm) apart, at right angles and between the *S.aureus* and *R.equi* streaks. The plates were incubated at 37°C for 24-48 hours. Enhanced synergistic reactions of *Listeria* strains with *R.equi* and/or *S.aureus* were regard as positive CAMP reactions (63, 150). The radii of the enhanced hemolytic zones were measured with a ruler from the edge of the bacterial growth streak to the outer perimeter of the hemolytic zones.

3.3.3 Xylose and rhamnose acidification tests

The procedure has been described previously (63). Purple broth base (DIFCO No.3)(DIFCO Laboratories, Detroit, USA) pH 6.8 was sterilized at 121°C for 15 minutes. Filter (0.45 μm Millipore membrane filters) sterilized L-rhamnose (Sigma Chemical Company, St.Louis, USA) and D-xylose (Sigma Chemical Company, St.Louis, USA) were added aseptically, to a final concentration of 1%. Ten ml of purple broth was aseptically inoculated with a loopful of *Listeria* cultures taken from 24 hour cultures that were grown on TSA-YE. The broths were incubated at 37°C for 2-14 days and visually monitored for a colour change (purple to yellow indicated a positive reaction) (64).

3.3.4 Antibodies

Primary antibodies, rabbit anti-α-Listeriolysin O antibodies (U. Kreft; pers. comm.[1]) and secondary antibodies, anti-rabbit Ig, horseradish peroxidase-linked whole antibodies (donkey) (Amersham, Buckinghamshire, England) were used.

3.3.5 Crude extraction of the Listeriolysin O toxin

The procedure used has been described (57) and involved the preparation of a ten-fold concentrated proteose-peptone broth [proteose-peptone No.3 (DIFCO Laboratories, Detroit, USA), 20 g; yeast extract (MERCK, Darmstadt, Germany), 5 g; $Na_2HPO_4.12H_2O$, 8.3 g; KH_2PO_4, 0.7 g; distilled water to 1000 ml]. Chelex 1000 sodium form (100-200 mesh) (Bio-Rad, California, USA) was added to 0.2% final concentration. This broth was stirred for 10 hours at room temperature. The resin was removed by Millipore filtration using a 0.45 μm filter. The Chelex-treated concentrated broth was further diluted in quartz-distilled water (1:10) and the pH was adjusted to 7.5 and the broth was sterilized. Filter sterilized glucose (2% final concentration) and sodium carbonate (0.25% final concentration) were added. One ml 24 hour brain heart infusion (OXOID Limited, Basingstoke, England) *Listeria* broth culture was aseptically pipetted into 9 ml of Chelex treated proteose-peptone broth and incubated for 24 hours at 37°C. After incubation cells were removed by centrifugation at 10000 x g for 20 minutes at 4°C (Beckman, model J2-21 centrifuge, rotor JA-20). Supernatant fluid (SNF) was aseptically transferred to pre-cooled, sterile centrifuge tubes. The protein was precipitated with 7% final concentration pre-chilled (-20°C) tri-choloroacetic

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acid (TCA) (MERCK, Darmstadt, Germany) at -20°C for one hour (82) and then centrifuged for 10 minutes at 8000 x g. The pellet was washed twice with 70 % pre-chilled (-20°C) ethanol (MERCK, Darmstadt, Germany) and was resuspended in two volumes of pre-chilled phosphate buffered saline pH 6.0. Protein concentrations were determined with the Bio-rad protein assay (Protein Assay Kit II, Bio-Rad Laboratories, California, USA). Samples were stored at -70°C. Samples were thawed on ice when required.

3.3.6 SDS-polyacrylamide gel electrophoresis

Protein samples were analyzed for the presence of a 60 kDa extracellular protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed in a linear gradient 5-20% w/v separation acrylamide gel containing a 5% stacking gel and also on 10% separating gels containing 5% stacking gels. The gels were run at 5 mA for 5 hours (91). Molecular weight markers (MW-SDS-70L Kit, Sigma Chemical Co., St.Louis, USA) and catalase (60 kDa) from bovine liver (British Drug House Limited, Biochemical Division, Poole, England) were used as markers. After electrophoresis proteins were visualised by staining with Coomasie brilliant blue R250 (MERCK, Darmstadt, Germany). Prior fixing of the protein on the gel for 2 hours in 5% formaldehyde before staining (J.W. Hastings; pers. comm.[1]) resulted in smaller losses of protein from the gels.

3.3.7 Western blotting

After SDS-PAGE, proteins were electro-transferred from unstained 10% gels as described (162). Transfer buffer consisted of 25 mM Tris,

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192 mM glycine, 20% methanol pH 8.3. Efficient transfer was obtained with the addition of 0.01% SDS to the transfer buffer. Proteins were transferred onto 0.45 μm Hybond-ECL nitrocellulose membrane (Amersham, Buckinghamshire, England) using a electrotransfer unit (GD-4, Pharmarcia, Sweden) for 3 hours at 1 A. The effectiveness of the transfer was assessed by staining the membrane with Ponceau S (Aldrich Chemical Company, Milwaukee, USA) for 5 minutes and destaining in four changes of distilled water.

3.3.8 Immunoblotting

Immunoblotting was performed according to the manufacturers instructions (Kit RPN 2109, Amersham, Buckinghamshire, England). The membrane was blocked overnight in Tris buffered saline pH 7.6 containing 0.3% Tween 20 (TBS-Tween) and 5-10% Carnation dried powder milk. The membrane was washed according to the manufacturers instructions, except that the washing was done at 40°C. Washing at the latter temperature reduced non-specific binding. Primary antibodies or pre-immune serum was diluted 1:1500 in TBS-Tween pH 7.6 and incubated overnight with the membrane at room temperature. Diluted secondary antibodies 1:2000 in TBS-Tween pH 7.6 were incubated with the membrane for 1 - 2 hours at room temperature. The final membrane wash was done in 0.01% SDS Tris-Tween buffer pH 7.6 for 15 minutes. Detection for the α-LLO toxin was done according to the manufacturers instructions using luminol chemiluminescent (Amersham, Buckinghamshire, England) and Agfa curix film (Agfa Gevaert, Leverkusen, Germany) or Hyperfilm-ECL (Amersham, Buckinghamshire, England).

3.3.9 Purification of chromosomal DNA from *Listeria*

Bacterial chromosomal DNA was isolated from *Listeria* by modifying a previously described method (123). A 1 ml overnight brain heart infusion broth (OXOID Limited, Basingstoke, England) culture of *Listeria* was aseptically pipetted into 99 ml of freshly prepared brain heart infusion broth and incubated at 37°C for 24 hours with gentle shaking. Cells were harvested by centrifugation, 4000 x g for 10 minutes (Beckman model J2-21, rotor JA-14). The pellet was resuspended in 10 ml of lysis buffer [25 mM Tris-HCl pH 8.0, 10 mM EDTA and 50 mM sucrose] and freshly prepared lysozyme (MERCK, Darmstadt, Germany) was added to a final concentration of 1 mg/ml. This mixture was incubated at 37°C for 30 minutes. Lysed cells were pelleted by centrifugation at 8000 x g for 10 minutes. The pellet was resuspended in 10 ml TEN buffer [50 mM Tris pH 8.0, 20 mM EDTA and 50 mM NaCl] and 50 μg/ml RNase (Boehringer Mannheim, Mannheim, Germany) were added and the mixture was incubated for 2 hours at 37°C. After incubation 2 ml of 10 % SDS and 4 mg/ml of freshly prepared pronase K (Boehringer Mannheim, Mannheim, Germany) were added. This mixture was incubated for 2 hours at 37°C. Further DNA extraction and purification were done according to standard methods (59). The purified DNA pellet was air dried for 2-3 hours and was resuspended in 500 μl of TE buffer [10 mM Tris pH 8.0 and 1 mM EDTA].

3.3.10 Labelling and origin of probes

Two oligonucleotide probes were designed from the Listeriolysin O gene (hly A) sequence published by Mengaud *et al.* (110). The probes were synthesized with an automated 6500 Autogen DNA synthesizer using standard phosphoramidite coupling method (136) and the synthesis was done at the Synthetic DNA Laboratory, Department of Biochemistry,

University of Cape Town, South Africa. The nucleotide sequence of the probes were:

1. LLO-1 = 5'- CCATggCACCACCAgCATCTCCgCCTgC - 3 '
2. LLO-2 = 5'- gCACTggTTTAgCTTgggAATggTggAgAACgg -3'

Probe LLO-2 contained the unique cysteine coding sequence which is highly conserved in genes that code for Streptolysin O and Pneumolysin toxins, whereas probe LLO-1 is from an unconserved region of the hly A gene and begins 112 bp downstream from the start codon (110). DNA probe hybridization was done according to standard methods (136). Probes were labelled with digoxigen-dUTP (DIG) according to the manufacturers instructions (DIG DNA labelling kit, Boehringer Mannheim, Mannheim, Germany). The approximate T_m for probes 1 and 2 were determined by standard methods (136) and were 69°C and 72°C, respectively.

3.3.11 Dot blot hybridization

Eight μl of a 0.1 μg/μl concentration of genomic DNA from each *Listeria* strain were treated with 2 μl of 1 N NaOH at 37°C for 15 minutes to denature the DNA into single strands (136). One μl of NaOH treated DNA was spotted onto a Nytran, 0.45 μm filter membrane (Schleicher and Schuell Incorporation, Dassel, Germany). Hybridization was done according to standard methods (136). The wet blots were developed according to manufacturers specifications using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Mannheim, Germany).

3.4 RESULTS

L.ivanovii (positive control) gave an elevated synergistic reaction (zone size was 7 mm) with *R.equi* in the CAMP test (Figs. 1a, b and c) and

The hybridization of chromosomal DNA of *L.monocytogenes* (control strain) and *Listeria* isolates DB121, SK135, L31, L35 and P73 with both probes were similar. Negative results were obtained for *L.welshimeri* and all the other *Listeria* isolates, which suggested that *L.welshimeri* and isolates AL1, AL2, AL3, AL5, P75, SK118 and SK133 did not possess the hly A gene (Figs. 4a and 4b).

Table 1 shows the correlation between the phenotypes and genotypes (CRX reactions, Immunoblotting and the dot blots). *Listeria* strains that were CAMP positive, acidified rhamnose and did not acidify xylose were regarded as pathogenic in accordance with a previous scheme (63).

3.5 DISCUSSION

A 91.7 % (11 of the 12 *Listeria* isolates) correlation between the CRX phenotype, presence of a 60 kDa protein that cross reacted with anti-α-LLO antibodies and the detection of the hly A gene by oligonucleotide probing (Table 1) was found. However, *L.ivanovii* (positive control) gave positive results in CAMP, SDS-PAGE, immunoblot and dot blot. Furthermore, it is well documented that *L.ivanovii* produces an antigenically similar the LLO toxin which is different in certain amino acids (87, 165), but since *L.ivanovii* did not acidify rhamnose in the CRX test (Table 1) it would imply that *L.ivanovii* is non-pathogenic according to the CRX classification system (63). Moreover, all *L.ivanovii* serotypes are rhamnose negative (63). Therefore, these results (Table 1) suggest that the CRX criteria (63) used for the classification of pathogenic *Listeria* species cannot be used to assess the pathogenicity of *L.ivanovii*.

FIG. 1a. CAMP reactions on sheep blood agar containing 5% sheep blood, after incubation at 37°C for 24 hours. (A) *S.aureus*; (B) *R.equi*; (1) *L.ivanovii* (positive control); (2) *L.welshimeri* (negative control); (3) *L.monocytogenes* (second positive control); (4) DB121; (5) SK118; (6) SK133 and (7) SK135.

FIG. 1b. CAMP reactions on sheep blood agar containing 5% sheep blood, after incubation at 37°C for 24 hours. (A) *S.aureus*; (B) *R.equi; (1) L.ivanovii* (positive control); (2) *L.welshimeri* (negative control); (3) *L.monocytogenes* (second positive control); (4) AL1; (5) AL2; (6) AL3 and (7) AL5.

FIG. 1c. CAMP reactions on sheep blood agar containing 5% sheep blood, after incubation at 37°C for 24 hours. (A) *S.aureus*; (B) *R.equi; (1) L.ivanovii* (positive control); (2) *L.welshimeri* (negative control); (3) *L.monocytogenes* (second positive control); (4) L31; (5) L35; (6) P73 and (7) P75.

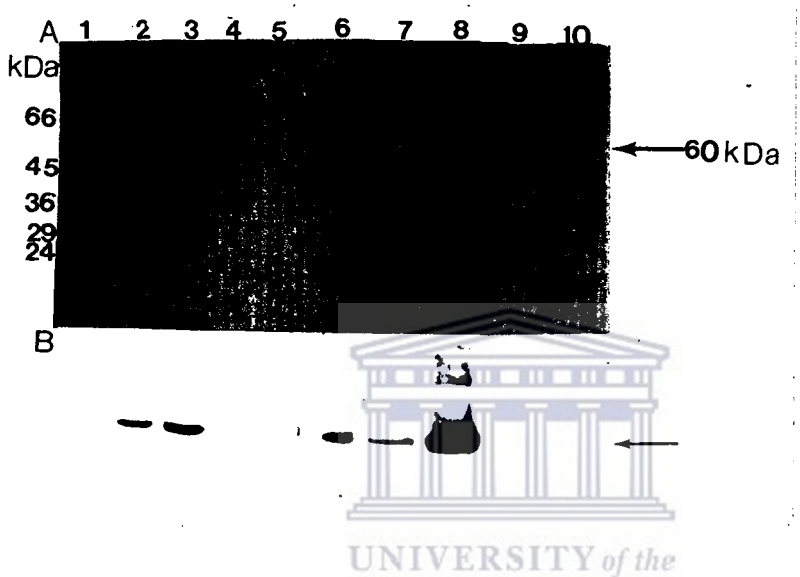

Fig. 2. (A) A 10% SDS-PAGE gel stained with Coomasie brilliant blue. Lanes: 1, molecular weight markers (MW); 2, *L.monocytogenes* (positive control); 3, SK135; 4, SK133; 5, SK118; 6, DB121; 7, L31; 8, L35; 9, catalase (60 kDa MW). About 3 μg of 7% TCA precipitated (82) extracellular protein was loaded for each strain and isolate, respectively. (B) A corresponding immunoblot with the arrow showing where positive signals were detected with anti-α-LLO antibodies.

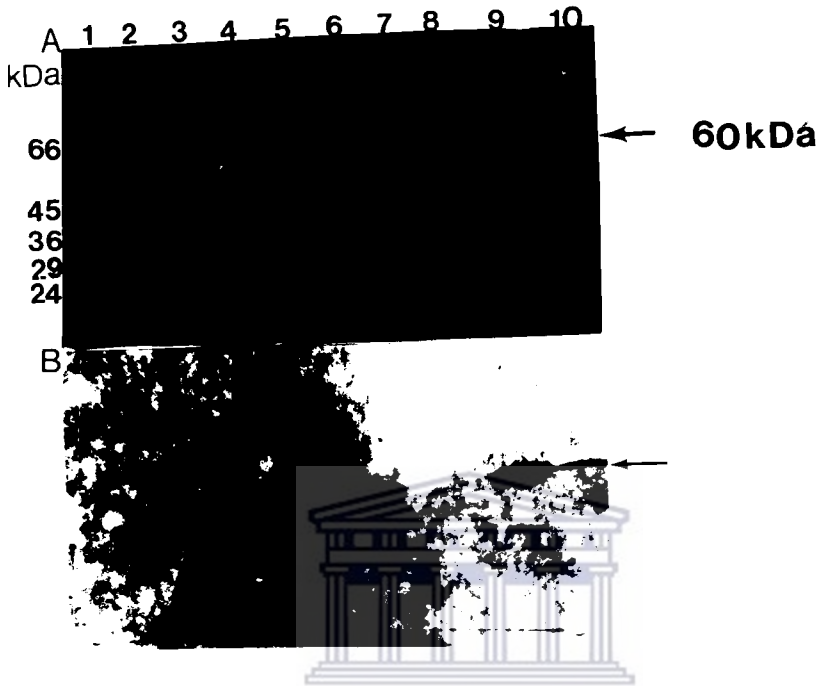

Fig. 3. (A) Coomasie brilliant blue stained 10% SDS-PAGE gel. Lanes: 1, molecular weight markers; 2, *L.ivanovii* (positive control); 3, *L.welshimeri* (negative control); 4, AL1; 5, AL2; 6, AL3; 7, AL5; 8, catalase (60 kDa MW); 9, P73; 10; P75. About 3 μg of 7% TCA precipitated (82) extracellular crude protein was loaded for each strain and isolate, respectively. (B) A corresponding immunoblot with the arrow showing where positive signals were detected with anti-α-LLO antibodies.

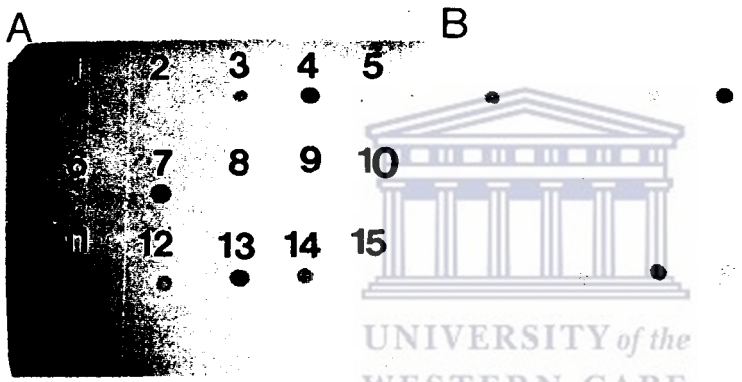

Fig. 4. Dot blot analysis of genomic DNA of *Listeria* spp. with LLO-1 (A) and LLO-2 (B) as follows: Lanes 1, *L.ivanovii* (positive control); lane 2, *L.welshimeri* (negative control); lane 3, *L.monocytogenes* (second positive control); lane 4, DB121; lane 5, SK118; lane 6, SK133; lane 7, SK135; lane 8, AL1; lane 9, AL2; lane 10, AL3; lane 11, AL5; lane 12, L31; lane 13, L35; lane 14, P73; lane 15, P75.

TABLE 1. Correlation of CRX tests, SDS-PAGE, immunoblot -and dot blot analysis

Listeria spp.	CAMP[a]		Rhamnose[b]	Xylose[c]	Pathogenic[d]	SDS-PAGE[e]	Immunoblot[f]	Dotblot[g]
	R.equi	*S.aureus*						
L.ivanovii	+ +	+	-	+	- *	+ + +	+ + +	+ + +
L.welshimeri	+	-	-	+	-	-	-	-
L.monocytogenes	-	+	+	-	+	+ + +	+ +	+
DB121	+ +	+	+	-	+	+ + +	+ +	+ + +
SK118	+ +	-	-	-	-	-	-	-
SK133	-	-	-	-	-	-	-	-
SK135	-	+	+	-	+	+ + +	+ + +	+ + +
AL1	-	-	-	+	-	-	-	-
AL2	-	-	-	-	-	-	-	-
AL3	-	-	-	-	-	-	-	-
AL5	-	-	-	-	-	-	-	-
L31	-	+	+	-	+	+	+	+
L35	+ +	+	+	-	+	+ + +	+ + +	+ + +
P73	+ +	-+	+	-	+	+ +	+ + +	+
P75	+	-+	+	-	+	-+	-+	-

[a]CAMP reaction zone sizes were grade as follow: + +,7-5mm; +, 2mm; +-, ≤ 1mm. The symbols used for [b]rhamnose and [c]xylose indicate: +, 100% positive reaction; -, negative reaction. For [d]pathogenic classification the symbols indicates: -, non-pathogenic; +, pathogenic; *, questionable result. For [e]SDS-PAGE, [f]immunoblot and [g]dot-blot the symbols indicate: + + +, + +, +, -+, 100% positive reactions; -, no reaction, but the intensity of the reactions as estimated visually were grade as follow: + + +, 100%; + +, 50%; +, 30% and -+, 5%. *L.ivanovii* and *L.monocytogenes* were used as positive controls and *L.welshimeri* was used as a negative control.

The arrow head hemolytic pattern produced by *L.ivanovii* with *R.equi* was previously observed (165) and this pattern differs from that produced by *L.monocytogenes*. Hence the CAMP reaction could be used as a rapid and reliable test to differentiate between these two species.

Synergistic hemolytic zones of the control *Listeria* strains and the *Listeria* isolates, with the exception of isolates P73 and P75, with *S.aureus* were about 3 mm and the zone with *R.equi* was generally > 3 mm. Skalka *et al.*(150) showed that pathogenic *L.monocytogenes* strains had higher hemolytic activity with *S.aureus* than with *R.equi*, results obtained in this study contradicts their findings, but is in agreement with similar findings (37, 87, 165).

Similar results (Table 1) to those of *L.monocytogenes* (positive control) were observed for isolates, DB121, SK135, L31, L35 and P73, which were previously identified as *L.monocytogenes* species. As expected *L.welshimeri* (negative control) was non-pathogenic (CRX) and did not produce a 60 kDa extracellular protein (Fig. 2). There were no signals from the hly A gene probes (Fig. 4) and no cross-reaction with rabbit anti-α-LLO serum (Fig. 3). Similar results were obtained with isolates SK118 (*L.innocua*), SK133 (*L.grayi*) and AL1, AL2, AL3, AL5 (*L.monocytogenes*). None produced a 60 kDa protein or hybridized to the probes and no signal was detected with rabbit anti-α-LLO serum which suggested that isolates AL1, AL2, AL3 and AL5 were non-pathogenic *L.monocytogenes* species. Although isolates AL1, AL2, AL3 and AL5 were identified as *L.monocytogenes* using the *Listeria* API system (BioMérieux, La Balme-les-Grottes, France), classical taxonomy should be used to confirm the identity of isolates AL1, AL2, AL3 and AL5. Nonetheless, the latter results are consistent with previously published results of Leimeister-wächter *et al.* (95) which proved that only pathogenic *L.monocytogenes* species

possessed the hly A gene, but contradicts results of Mengaud *et al.* (110) which detected hly A internal regions in all *L.monocytogenes* species, including non-pathogenic species. Isolate P75 which was previously identified by the *Listeria* API identification system (BioMérieux, La Balme-les-Grottes, France) as *L.monocytogenes*, produced a 2 mm synergistic hemolytic zone with *R.equi* in the CAMP test and a band intensity of about 5% for the 60 kDa extracellular protein was visible on the SDS-PAGE gel. A positive signal was detected with anti-α-LLO antibodies (Fig. 3), but no signal was detected with either of the two probes (Fig. 4).

Therefore, the results obtained for *L.monocytogenes* P75 might corroborate a previous study by Parrius *et al.* (117) which found that only 2 out of 28 virulent *L.monocytogenes* strains tested produced the classical α-LLO toxin and led them to postulate that there were two immunologically distinct LLO toxins, α-LLO and β-LLO toxin. It was speculated that the reason they did not detect α-LLO toxin was probably because some of the strains produced very low concentrations of the toxin due to culturing media influences (56) and secondly, doubts were raised about the specificity of the antibodies used, since they were prepared from a crude extract. It was also suggested that the media which were used; in this study were best suited for α-LLO toxin production (56). However, more recently, the production of two distinct LLO toxins appears to be supported by DNA hybridization studies of Mengaud *et al.* (110). The only omission was that the probe they used lacked the cysteine coding sequence, which is highly conserved among other cytolysins (87, 95). One of the probes used in this study, probe LLO-2 consisted entirely of this region, but still no hly A gene was detected in the genomic DNA of isolate P75 and therefore, the results obtained for isolate P75 seem to support the existence of two distinct LLO toxins (110, 117), but further research is required to confirm the latter.

3.6 CONCLUSION

There was 91.7% correlation between pathogenesis as measured by the CRX tests and the production of an extracellular protein of approximately 60 kDa, for 11 out of 12 *Listeria* isolates. This protein was immunologically similar to α-LLO toxin since it cross-reacted with anti-α-LLO antibodies. This correlation was, furthermore, confirmed by detecting the hly A gene in virulent *Listeria* species only. Non-virulent *Listeria* strains were non-pathogenic (CRX), did not produce a 60 kDa extracellular protein, no signals from the hly A gene probes were detected and no cross-reaction with rabbit anti-α-LLO serum occurred. Therefore, our results, with the exception of isolate P75 are in agreement with the fact that only pathogenic *Listeria* species produce the α-Listeriolysin O toxin (87, 95, 129, 155).

Moreover, two *L.monocytogenes* strains (isolates DB121 and SK135) isolated from raw milk and 37.5% (three out of eight) *L.monocytogenes* strains (L31, L35 and P73) isolated from raw chicken were pathogenic. In total five out of twelve (41.7%) of the *Listeria* isolates from raw milk and raw chicken were pathogenic.

Since this is the first study of its kind that investigated the virulence nature of *Listeria* isolated from raw milk and raw chicken in the Western Cape, South African, it could serve as a bases for a more comprehensive study into the virulence of South African *Listeria*. A more comprehensive study could serve to confirm these findings, namely that *Listeria* isolated from raw milk and raw chicken in the Western Cape, South Africa possess the same major virulence factor, Listeriolysin O as compared with international isolates of *Listeria.* A study into the latter suggested area of research will contribute to finding out the reason for high incidence of *Listeria* in dairy

products in South Africa (74, 75), whilst no foodborne associated incidence of listeriosis occurred in South Africa, whereas in America and Europe high incidence of foodborne associated listeriosis occurred since 1981 (43).

The results on the correlation of α-Listeriolysin O toxin by immunoblotting and the hly A oligonucleotide probing, using two different probes, for *L.monocytogenes* P75 are interesting since the CRX phenotype of isolate P75 was typical of a pathogenic *L.monocytogenes* strain (63), except that no hly A gene could be detected. Further work needs to be done to establish whether isolate P75 produces a different "hemolysin", which is similar in certain aspects (immunologically, size and β-hemolysis) to classical α-LLO toxin, but probably not coded for by the same hly A gene.

In general the two oligonucleotide probes appeared to be highly specific for pathogenic *Listeria* strains and could, upon further testing, be used for rapid detection of pathogenic *Listeria* species in food.

CHAPTER 4

4 GENERAL CONCLUSION

Listeria monocytogenes is a foodborne pathogen that is the etiological agent of the life threatening disease listeriosis (54, 151). Since 1981 there has been an alarming increase in food-associated listeriosis in Europe and America and furthermore, this bacterium was isolated from various foods (dairy products, meat, fish, vegetables and fruits) (43). Among the various foods; dairy products (4, 38, 65, 104, 106) and poultry (59, 70) were regularly found to be contaminated with *Listeria*, but dairy products were implicated in food-associated outbreaks of listeriosis (22, 48, 97) whereas poultry was not implicated. Furthermore, dairy product associated listeriosis outbreaks were accompanied by high human mortality and morbidity rates (43). Even more disconcerting to the consumers and food manufacturers was that pasteurization, freezing and addition of preservatives such as NaCl, nisin, nitrite and nitrate were reported as being ineffective in the successful elimination or control of *L.monocytogenes* in food (40).

By contrast to America and Europe, in South Africa one outbreak of listeriosis (73) and sporadic cases of listeriosis (1, 126) have been reported, but none of these reported cases of listeriosis were linked to consumption of *Listeria*-contaminated foods. Furthermore, the virulence nature of international isolates of *Listeria* are well studied and sufficient evidence indicates that the pathogenesis of these *Listeria* species is linked to the production of a major virulence factor α-Listeriolysin O (14, 28, 110), whereas in South Africa and more particularly in the Western Cape, South Africa nothing is known about the virulence nature of *Listeria* present in

raw milk and raw chicken. Therefore, the objective of this study was to isolate *Listeria* from raw milk and raw chicken in the Western Cape and to determine the virulence traits of these *Listeria* isolates.

Two raw milk samples, collected from bulk tanks in the Western Cape, were analyzed for *Listeria* by the United States of America's Department of Agriculture Food Sanitation Inspective Services (FSIS) and the United States of America's Food and Drug Administration (FDA) methods (93, 99). Four *Listeria* isolates (DB121, SK118, SK133 and SK135) were isolated from both raw milk samples. Isolates DB121 and SK135 were identified as *Listeria monocytogenes* by morphological and biochemical tests as well as by the *Listeria* API identification system and isolates SK133 was identified as *L.grayi*. Isolate SK118 could only be identified as *L.innocua* using the *Listeria* API identification system and not by morphological and biochemical tests; since it hydrolysed casein, whereas none of the known *Listeria* species exhibit this characteristic (145). Eighteen raw chicken samples, taken from one poultry processing plant in the Western Cape, were analyzed by a modified FSIS method and eight isolates (isolated from 8 samples) were identified as *L.monocytogenes* using the *Listeria* API identification system. *L.monocytogenes* (isolates DB121 and SK135) was isolated from both raw milk and *L.innocua* (isolate SK118) and *L.grayi* (isolate SK133) were isolated from only one raw milk sample (Chapter 1).

The virulence nature of these 12 *Listeria* isolates which were isolated from raw milk (4 isolates) and raw chicken (8 isolates) in the Western Cape, South Africa, was determined by the CAMP reaction, rhamnose and xylose acidification (CRX) tests, detection of a 60 kDa α-Listeriolysin O toxin by sodium dodecyl sulphate polyacrylamide gel electrophoresis, Western blotting and immunoblotting and by oligonucleotide probing, using two different oligonucleotide probes, to detect the presence of the hly A gene.

In the case of *L.monocytogenes* NCTC 7973 (positive control) a 100% correlation was found between pathogenesis as determined by the CRX test, detection of an immunologically similar α-LLO toxin and the detection of the hly A gene. The converse was true for *L.welshimeri* ATCC 5334 (negative control), this bacterium was non-pathogenic and no α-LLO toxin nor the hly A gene was detected. Six *Listeria* isolates were CRX positive and expressed a 60 kDa protein that gave a positive signal when reacted with anti-α-LLO antibodies. Genomic DNA samples of 5 of these 6 isolates also showed strong hybridization signals when reacted with hly A oligonucleotide probes. There was 91.7 % (11 of the 12 isolates) correlation between CRX phenotype, presence of a 60 kDa protein that cross reacted with anti-α-LLO antibodies and the detection of the hly A gene by oligonucleotide probing. With the exception of isolate P75 the results were consistent with the fact that only virulent *L.monocytogenes* strains, as determined by the CRX test, produced a 60 kDa extracellular toxin (evident from its cross-reaction with anti-α-LLO antibodies) and possessed the hly A gene (hybridization with two oligonucleotide probes).

Isolate P75 produced a 60 kDa extracellular protein (Fig. 3) and produced hemolysis in the CAMP test (Fig. 1c). This protein cross-reacted with rabbit anti-α-Listeriolysin O (Fig. 3b), but the hly A gene was not detected with either of the two probes (Fig. 4). Although this result may corroborate previous speculations of two possible distinct toxins (110, 117), further work needs to be done to confirm this.

Since it was not the aim of this study to determine the incidence of *Listeria* in raw milk and raw chicken in the Western Cape, it is not possible to say whether the occurrence of *Listeria* in raw milk from bulk collection tanks, and in raw chicken from one poultry processing plant, in the Western Cape is significant since only two raw milk samples and 18 raw chicken samples were tested for *Listeria.* Hence a more comprehensive study should be conducted to determine whether the incidence of *Listeria* in raw milk and

raw chicken in the Western Cape is significant. Nonetheless, the objectives of this study was achieved since four *Listeria* species from raw milk and eight *L.monocytogenes* from raw chicken milk were isolated and it was proven that there was 91.7% (11 out 12 isolates) correlation of CRX tests, detection of a 60 kDa α-Listeriolysin O toxin and detection of the hly A gene between the 12 *Listeria* species isolated from raw milk and raw chicken in the Western Cape, South Africa and the reference *Listeria* strains. Therefore, these 12 *Listeria* isolates from raw milk and raw chicken in the Western Cape, South Africa corresponded to the reference *Listeria* strains with regard to the possession of an important virulence factor α-Listeriolysin O toxin.

Furthermore, 41.7% of these *Listeria* isolates were found be pathogenic, it thus emphasizes the need for more intensive research into the prevalence of virulent *L.monocytogenes* in raw milk and raw chicken in order to get a more comprehensive indication of whether the occurrence of virulent *Listeria* species in these foods are significant and whether the presence of this bacterium in raw milk and raw chicken poses a serious health threat to consumers. Raw milk contaminated with *Listeria* if not properly pasteurized (38, 47, 48) and chicken containing *Listeria* if not properly cooked (141) prior to consumption by humans could lead to the deathly disease listeriosis.

In conclusion this is the first study of its kind that investigated the virulent nature of *Listeria* isolated from South African foods and the results on the correlation of α-Listeriolysin O toxin by immunoblotting and the hly A oligonucleotide probing, using two different probes, for *L.monocytogenes* P75 are interesting and future research should be aimed at isolation and characterization of the 60 kDa "hemolysin" produced by isolate P75 and to determine whether it is a "toxin" and the extent of similarities with the

classical α-LLO toxin. Research in the area suggested above will contribute to finding out whether there are two different toxins and the relationship of these toxins with regard to pathogenicity of *L.monocytogenes*. Secondly the two probes appeared to be highly specific for pathogenic *Listeria* strains and could, upon further testing, be used for rapid detection of pathogenic *Listeria* species in food.

CHAPTER 5

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