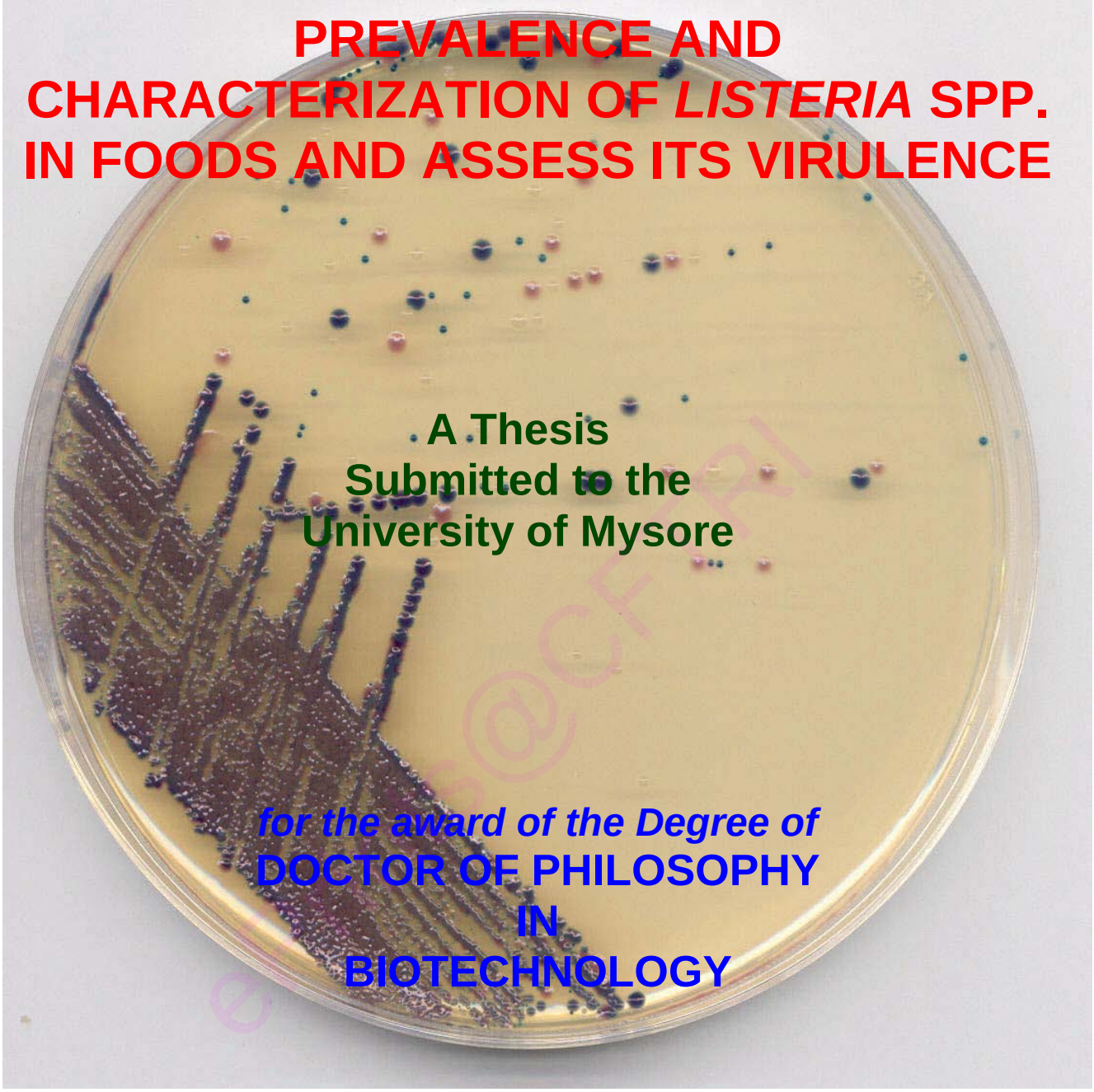
# PREVALENCE AND CHARACTERIZATION OF *LISTERIA* SPP. IN FOODS AND ASSESS ITS VIRULENCE



**A Thesis**
**Submitted to the**
**University of Mysore**

***for the award of the Degree of***
**DOCTOR OF PHILOSOPHY**
**IN**
**BIOTECHNOLOGY**


***By***
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**December 2008**

**Dedicated**

***to my***

# PARENTS

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## CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"PREVALENCE AND CHARACTERIZATION OF *LISTERIA* SPP. IN FOODS AND ASSESS ITS VIRULENCE"** submitted by Mrs. H.A. Vageeshwari for the award of degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE**, is the result of the research work carried out by her in the Departments of Human Resource Development and Food Microbiology, Central Food Technological Research Institute, Mysore under my guidance and supervision during the period of Ph.D. programme. This has not been submitted earlier, either partially of fully for any other degree or fellowship.

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## DECLARATION

I hereby declare that the thesis entitled **"PREVALENCE AND CHARACTERIZATION OF *LISTERIA* SPP. IN FOODS AND ASSESS ITS VIRULENCE "** submitted to the **UNIVERSITY OF MYSORE**, for the award of degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY**, is the result of the research work carried out by me under the guidance of **Dr. M.C. Varadaraj**, Head, Human Resource Development, CFTRI, Mysore during the entire tenure of my CSIR-SRF Fellowship programme. I further declare, that the results presented in this Thesis have not been submitted for the award of any other Degree or Fellowship.

(H.A. VAGEESHWARI)

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*Vageeshwari H.A.*

# Thesis Synopsis

# Introduction

# Review of Literature

# Object & Scope of the Study

# Experimental Plan, Results and Discussion

# Summary and Conclusion

# Bibliography

FT/HRD/Ph.D./2008

December 31, 2008

**Dr. M.C. VARADARAJ**
Head, Human Resource Development

## ATTENDANCE CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"PREVALENCE AND CHARACTERIZATION OF *LISTERIA* SPP. IN FOODS AND ASSESS ITS VIRULENCE"** submitted by **Mrs. H.A. Vageeshwari** for the award of degree of **DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE**, is the result of the research work carried out by her in the Departments of Human Resource Development and Food Microbiology, Central Food Technological Research Institute, Mysore under my guidance and supervision during the period of Ph.D. programme.

(M.C.VARADARAJ)
Research Guide

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**Synopsis of Ph.D. Thesis**

# PREVALENCE AND CHARACTERIZATION OF *LISTERIA* SPP. AND ASSESS ITS VIRULENCE

The broad spectrum of foodborne infections has changed dramatically over the years, as well-established pathogens have been controlled or eliminated and new ones have emerged. The burden of foodborne disease remains substantial. The spectrum of foodborne pathogens includes a variety of enteric bacteria, aerobes and anaerobes, viral pathogens and parasites as well as marine dinoflagellates. *Listeria* is one of the pathogenic bacteria emerged in 1980's in the scene of public health causing serious foodborne outbreaks in western countries. This pathogen viewed as emerging one due to its ubiquitous nature, tolerance to adverse conditions and typical characteristics of being able to survive and even multiply in refrigerated food products. In view of the public health significance of *Listeria* spp., reviews have been documented on varied aspects (Ryser and Marth, 1999).

*Listeria* is widely distributed in nature and can be readily isolated from soil, water, sewage, green plant material, decaying vegetation and numerous species of birds and mammals, including humans (Gray and Killinger, 1966). *Listeria monocytogenes* is a facultative human pathogen commonly found in the environment and can exist in an intracellular state within monocytes and neutrophils (Schuchat et al. 1992). According to Bergeys Manual of Systematic Bacteriology, *Listeria* has been placed as a genus in the family Listeriaceae

under the Order: Bacillales. *Listeria* are small (1.0-2.0 x 5 µm) Gram positive, facultative anaerobic, rod shaped bacterium. *Listeria* is a psychrotropic that can survive for long periods in cold storage. It can grow in a wide range of temperatures from $-1.5^0$C to $50^0$C and pH of 4.3 to 9.6 (Farber et al. 1989; Hudson et al. 1994).

*Listeria* can be isolated by conventional selective isolation methods (like FDA, USDA and others), which involves steps of enrichment and plating on selective isolation medium. Nucleic acid based methods such as PCR, RT-PCR, multiplex PCR, Nested PCR, RFLP and RAPD as well as immunological methods like ELISA, ELFA and immuno-precipitin have enabled in rapid and specific detection of these organisms, both in pure culture system and in certain foods.

Studies relating to incidences of *Listeria* spp. in foods in India are few, with much emphasis on sea foods (Fuchs and Surendran, 1989; Kamat and Nair, 1994; Jayashekaran et al. 1996; Karunasagar and karunasagar, 2000). Few studies have been carried out to investigate the occurrence of *Listeria* spp in milk and milk based products (Pednekar et al. 1997; Warke et al. 2000; Barbuddhe et al. 2002), vegetables (Pingulikar 2001) and meat from Indian market (Kamat and Nair, 1994; Barbuddhe, 2002). The reports on neonatal listeriosis (Thomas et al. 1981) and listeriosis during pregnancy (Gupta et al. 2003) and the presence *L.*

*monocytogenes* in clinical samples (Dhanashree et al. 2003) has emphasized the documentation of prevalence of *Listeria* spp. in the present Indian scenario.

In the background of information documented in literature about public health significance of these *Listeria* species, the present study was done to detect the prevalence of *Listeria* spp. in different foods. Antibody based and DNA based techniques were developed for rapid detection and the virulence of native isolates were assessed using *Drosophila* model. The data generated from the above investigations are presented in Ph.D. programme along with introduction, review of literature and summary & conclusion and Bibliography.

In this background, it is desired that studies should primarily focus on:

- Prevalence of *Listeria* species including those of *L. monocytogenes* in foods
- Develop detection systems, which can detect large number of *Listeria* species over that of *L. monocytogenes*
- Assess the virulence factors, which may exist among non-*L. monocytogenes* cultures

The major aspects covered in various sections of review of literature are summarized below.

- ❖ Foodborne pathogens
- ❖ *Listeria* and its species

- Methods of isolation
- Virulence determination
- Incidence in food

In the third chapter, object and scope of the study has been discussed. The fourth chapter involves four sub-chapters, which focuses on experimental plan, results and discussion.

**PREVALENCE OF *LISTERIA* SPECIES IN DIVERSIFIED FOODS**

Prevalence of *Listeria monocytogenes* and other *Listeria* spp. in different foods like milk and milk based products, meat and meat based products, vegetables and vegetable based were assessed, using non-selective cold enrichment technique. The presumptive isolates were further confirmed by morphological, biochemical and cultural characteristics.

**IMMUNOLOGICAL METHODS FOR THE DETECTION OF *LISTERIA MONOCYTOGENES***

Listeriolysin O was purified by *L*. *monocytogenes* cultures and characterized. The pure LLO was used in production of antibody in rabbit and poultry. The produced antibody was used in the detection of *L. monocytogenes* in pure form and from artificially contaminated milk and market samples. Immunobeads were prepared by coating antibody to protein A beads and immunocapture of *L*. *monocytogenes* was carried out from milk.

**MOLECULAR METHODS FOR THE DETECTION OF *LISTERIA* SPP.**

Primers were designed for the detection of all pathogenic *Listeria* and different spp. in particular. Uniplex, multiplex, nested and immunoPCR were carried out for the detection of *L*. *monocytogenes* in pure cultures and in artificially contaminated milk. The native isolates were confirmed by PCR technique.

**ASSESSING THE VIRULENCE TRAITS IN *LISTERIA* SPECIES**

Native *Listeria* isolates were assessed for virulence using *Drosophila* model. The *Drosophila* was established as a model for pathogenicity assessment in respect of *Listeria* cultures and other Gram positive and Gram negative bacteria.

The thesis concludes with a brief summary and conclusion, followed by documentation of relevant references from literature.



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Guide

## 1.0 INTRODUCTION

Foodborne infections and intoxications are of serious health concern. Over the years, microbial food safety has emerged as the foremost runner in positioning food industry on a global platform. The impact of foodborne health hazards is almost uniform, with no boundary limitations in respect of age, sex, economic status and other influencing factors on human populations. Preventing foodborne pathogenesis is a multifaceted process. The general strategy of prevention is to understand the mechanisms by which contamination and disease transmission can occur well enough to interrupt them. An outbreak investigation or epidemiologic study should go beyond identifying a suspected food. The need of the situation is methods to recall implicated foods and define the chain of events that allowed the contamination to occur and reach risk-causing levels.

Meaningfully, monitoring of foodborne disease requires an effective surveillance system at the local, national and international levels. To date, resources have been limited for most countries and regions to do this and the current knowledge is based, for the most part, on passive reporting mechanisms. The statistics of pathogen causing illness is mainly calculated based on the existing database, which is restricted to a few countries. Even though special epidemiological and outbreak investigations are necessary for the identification and controlling foodborne diseases, the laboratory isolation methods and required expertise gives less opportunities to cope with the global scenario of microbial food safety.

The epidemiology of foodborne disease is changing and many newer dimensions are being projected in the area of pathogenic bacterial species. In the mid 1980s, *Listeria monocytogenes* and few other related species emerged as organisms of serious health concern through reported outbreaks of listeriosis. *Listeria*

ingested in the contaminated food a common transient colonizer in the gastrointestinal track promotes infection. Diagnosis of listeriosis is not possible unless the organism enters the blood stream. In healthy human beings, infection can give rise to atypical meningitis, febrile gastroenteritis characterized by fever and watery diarrhoea of 2-3 days duration, bacteriemia characterized by acute febrile illness, often with myalgias, arthritis, headache and backache or remain asymptomatic. Clinical disease by *Listeria* is mainly associated with ingestion of variety of food products. USDA-FSIS monitoring programme has shown a wide range of raw meat, meat products like jerky, sausages, roasted, cooked or cornered beef, hot dogs and hams contaminated with *Listeria*. Since *Listeria* is ubiquitous in environment, the fresh produces have been shown to be the sources.

Based on documented literature reports, listeriosis outbreaks and prevalence of *Listeria* in foods have been on a higher proportion in USA and few of the European countries. In the context of emphasis on microbial food safety, the scenario in India does not imply that issues relating to *Listeria* spp. in foods are on a very low key. Detection of *Listeria* is still a challenging task for food microbiologists and Food Regulatory Personnel and Authorities. The detection should be simple to perform by even non-specialized personnel and sensitive enough to detect low numbers of specific and targeted pathogen as well as differentiate very closely related species. Conventional identification of *Listeria* involving culture methods based on selective enrichment, plating and characterization are widely recommended by regulatory bodies and even today continue to be considered as golden standards.

Considering the public health significance of *L*. *monocytogenes*, it is essential to know the important identifying characteristics. Strains of *L. monocytogenes* are Gram positive, non-sporing, facultatively anaerobic rods, which appear coccoidal in older cultures. The organism is catalase positive and oxidase negative and grows in

the range of -0.4 to 50°C and thrives best under anaerobic to microaerophilic conditions, wide range of temperatures and pH levels. On the blood agar, β-haemolysis is produced. The haemolysin acts synergistically with the β-haemolysin of *S. aureus* on sheep erythrocytes, the substance mediating this effect is known as CAMP factor after Christie, Atkins and Munch-Petersen. The organism possesses peritrichous flagella, which gives characteristic tumbling motility. Strains of *L. monocytogenes* are widely present in the natural habitats and ecosystem.

Isolation and detection of *L. monocytogenes* from environments including foods with a competitive microflora offers a challenging task for the microbiologists due to background microflora naturally occurring in the foods. Direct plating, cold enrichment, selective enrichment and several rapid methods all can be used in various combinations to detect *L. monocytogenes* in food, clinical and environmental samples. Early attempts to isolate small numbers of *Listeria* from samples containing large populations of indigenous microflora relied on direct plating and often ended in failure. Cold enrichment procedure was introduced as an alternative method to isolate *L. monocytogenes* from highly contaminated samples. Although this method has contributed much to our present-day knowledge concerning the epidemiology of listeriosis, the prolonged incubation period necessary to obtain positive results has been a serious disadvantage. Major improvements in selective enrichment and plating media have since decreased time of analysis from several months to less than 1 week. Alternatively, antibiotics and other chemicals have been used in order to shorten the time required for isolation. The aim of using these reagents is to develop a selective direct plating medium which suppresses the growth of competing microflora and support the formation of colonies of *L. monocytogenes* in an incubation period of 24-48 h.

On many occasions, the conventional isolation protocols lack clear-cut specificity and often lead to false positive detection of *L. monocytogenes* and related species. Viewing a few of these drawbacks, several molecular and immunological methods have been developed and are in commercial use. Considering the primary trait of virulence in the culture of *L*. *monocytogenes*, DNA based molecular methods have targeted the haemolysin gene of this bacterial species, which is known to encode the toxin, listeriolysin O. As this toxin has a good secretory ability, the same has been utilized to develop immunological methods based on antibody production.

Heightened world-wide interest in foodborne listeriosis coupled with the advent of mandatory HACCP programs for meat and seafood products in the United States has led to development of more reliable commercial screening methods for *Listeria*. Two protocols developed in the United States by the FDA and USDA-FSIS have emerged as 'standard methods' to isolate *L. monocytogenes* from dairy foods, seafoods, vegetables and meat and poultry products, respectively. Despite widespread use of these methods in the United States, Canada and Western Europe, both procedures are still plagued with difficulties that include the inability to isolate *Listeria* from all positive samples as well as difficulties in recovering injured cells. In response to these concerns, the USDA-FSIS and FDA protocols have been modified to enhance recovery of injured *Listeria*. Working in co-operation with the International Dairy federation (IDF), other Official European Agencies have developed somewhat similar protocols, which are partially based on current FDA methodology.

An increase in the number of listeriosis outbreaks and the discovery of *L. monocytogenes* in raw and ready-to-eat meat, poultry, milk and milk products, seafoods and vegetables have underscored the need for continuing studies on foodborne *Listeria* spp. Listeriosis and *L. monocytogenes* continue to be of world-wide interest in the food chain establishment.

# 2.0 REVIEW OF LITERATURE

## 2.1 FOODBORNE PATHOGENS

Foodborne diseases are considered as a major cause of health problems in today's life. The broad spectrum of foodborne infections has changed dramatically over time, with well-established pathogens being controlled or eliminated and new ones emerging, thus the burden of foodborne diseases remaining substantial (Tauxe, 1997). The disease threat may be due to the increase in human population, globalization of food supply, international travel and trade without any cross-country barriers, microbial adaptation and changes in the food production system, as well as human demographics and behaviour. Contamination may occur at any point in the food chain, right from the field to consumption passing through several unit operations. Changes in farming practices with larger operations and faster throughput, the drive to increase profit by recycling animal materials, difficulty in disposal of manure and other related issues have lead to increased livelihood of contamination of raw materials.

Contamination typically occurs early in the production process, rather than just before consumption. The evolving technologies for food production, processing and distribution have lead to the spread of new pathogens (Farber, 1991). Infectious and toxigenic pathogens transmitted through food have been recognized for over centuries. Some known pathogens have only recently been shown to be predominantly foodborne, such as *Listeria monocytogenes* and *Campylobacter jejuni*. Centre for Disease Control, USA has recognized more than 250 foodborne diseases caused by bacteria, viruses and parasites. Many pathogens including *Salmonella* spp., *Escherichia coli* O157:H7, *Campylobacter* spp. and *Yersinia enterocolitica* have reservoirs in healthy food animals, from which they spread to an increasing variety of foods.

Bacterial incidences measured by disease outbreaks have recognized *Escherichia coli, Staphylococcus aureus, Campylobacter jejuni, Clostridium perfringens, Salmonella* spp. and *Listeria monocytogenes* as the emerging pathogens (Bacteriological Analytical Manual, 2003). On an average, food posioings reported annually in USA (Centre for Disease Control, USA, 2006) covers 76 million illness, 325000 hospitalizations and 5000 deaths each year. In other countries, there appears to be a lacuna in the reporting and documentation systems, except for certain sporadic reports here and there.

## 2.2 LISTERIA AND ITS SPECIES

### 2.2.1 Occurrence

*Listeria monocytogenes* is widely distributed in the environment. Organism has been isolated from soil (Weis et al. 1975), water, sewage (Watkins et al. 1981) and decaying vegetation (Welshimer, 1965). Primary habitats are soil and decaying vegetation, wherein the organism leads a saprophytic life (Welishimer, 1960). Soil serves as a reservoir for further transmission to animals and humans. The relationship between ingestion of *Listeria* contaminated silage, infection in dairy animals like cow, sheep and goat has been documented (Fenlon, 1999). Faeces shed by these animals along with silage are the vehicles for primary infection in ruminants and asymptomatic shedding of *Listeria* in milk transmits the disease to humans (Arimi et al.1999). Humans also serve as the reservoir for the organism. *Listeria monocytogenes* has been isolated from 5 to 10% of stools of healthy adult humans (Schuchat et al. 1993). *Listeria* is ubiquitous and associated with variety of mammals, birds, fish, amphibians and insects. Due to their ubiquity, organism entering food, food production and food processing environment is quite possible. Nearly, 15% to 70% of raw milk, vegetables,

unpasteurized milk, soft-cheese and meat contamination with *L. monocytogenes* has been documented over the past one decade and plus (Farber, 1991).

### 2.2.2 Epidemiology

Estimates of listeriosis are limited to invasive disease as *Listeria* is asmptomatic in the normal carriers. The first reported human case occurred in a soldier of the First World War, who suffered from meningitis. Since the time of First World War till 1950, only a few human cases were reported. However, at present, hundreds of cases are reported every year (Rocourt and Cossart, 1997). Listeriosis is an a typical foodborne disease of major public health concern because of the severity and the non-enteric nature of the disease (meningitis, septicaemia and abortion), a high fatality rate (around 20-30% of cases), a long incubation period and a predilection for individuals who have underlying conditions, which lead to impairment of T-cell mediated immunity.

The emergence of listeriosis is the result of complex interactions between various factors reflecting changes in social patterns. These factors include (I) medical progress and consequent demographic changes, such as the increasing proportion of immuno-comprised people, (ii) change in primary food protection, (iii) change in food habits and (iv) changes in food handling and preparation practices.

Food Net data indicates Listerial infections are sporadic. During 1980's the incidence of listeriosis was estimated approximately to be 7.4 cases per million population or about 1,850 cases per year with about 90% mortality (Ciesielski et al. 1988; Gellin et al. 1991). In the year 1993, there was a decrease (4.4 cases per million population) due to food industry regulations (Tappero et al. 1995). Recent reports of CDC of USA (2006) have shown the decline in listeriosis to 2.7 cases per million

population. Even though the reported incidences of *Listeria* are very low, the mortality is very high up to 90%. Incidence of listeriosis in the exposed population during the outbreaks has been estimated to be around 5 cases per one hundred thousand population (Bille et al. 2003).

The outbreaks of foodborne *Listeria* have been recognized since 1981. During 1980's, the outbreaks characterized by bacteremia and meningitis showed listeriosis as an invasive disease. Several outbreaks characterized by febrile gastroenteritis have also been recorded. The first and major outbreak was observed in Canada during 1981. Coleslaw, a product of cabbage and carrot had taken the lives of 18 people (2 adults and 16 fetal or newborn) out of 41 cases.

Mexican style cheese consumption caused 48 deaths in Los Angeles in 1985, chocolate milk in Illinois in1995, Hotdogs and Deli meats in several states during 1998 and 1999 are the few reports of listeriosis as invasive disease (CDC of USA, 1998; 1999). These outbreaks involved *L. monocytogenes* serotype 4b. Few uncommon *L. monocytogenes* in deli turkey also caused listeriosis during 2002 (CDC of USA, 2002).



**2.2.3 Taxonomy**

The genus *Listeria* is placed in the *Clostridium* sub-branch of Gram positive bacteria, under the order Baciliaceae, based upon the low G+C content of its genome. Genus *Listeria* contains six species: *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii* and *L. grayi.* The species of *L. grayi* consists of two subspecies namely *L. grayi* subsp. *grayi* and *L. grayi* subsp. *murrayi*. Similarly, *L. ivanovii* consists of two subspecies namely *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londonensis* (Bacteriological Analytical Manual, 2003, Bergey's Manual of Systematic Bacteriology

Vol 2, 9th edition, 1986). Based on DNA/DNA hybridization, 16S rRNA cataloging and reverse transcriptase sequencing of 16S and 23S rRNA, the genus embraces two closely related, but obviously distinct lines of descent. One contains *L. grayi* and the other *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri* and *L. seeligeri* (Collins et al. 1991).

Murray in 1926 definitively described *Listeria monocytogenes* for the first time in conjunction with an outbreak of disease among laboratory rabbits at Cambridge University. Pathogenicity of the organism was established by isolating it from blood of the infected rabbits and reinfecting healthy organism. The organism was named *Bacterium monocytogenes* as it caused mononucleosis-like infection (Murray, 1926). In 1927, Pierie documented a similar outbreak in South Africa involving wild gerbils. This disease was termed tiger liver disease and named the organism *Listerella hepatoltica* in honour of Lord Lister (Piere et al. 1927).

Both the organisms were identified to be similar and named *Listeria monocytogenes* prior to this organism and were well recognized in infecting ruminants and humans. In 1929, Gill described the illness in sheep called circling disease caused by *L. monocytogenes* (Gill et al. 1937). Same year, Nyfeldt isolated the organism from human patients suffering from mononucleosis like infection (Nyfeldt et al. 1929).

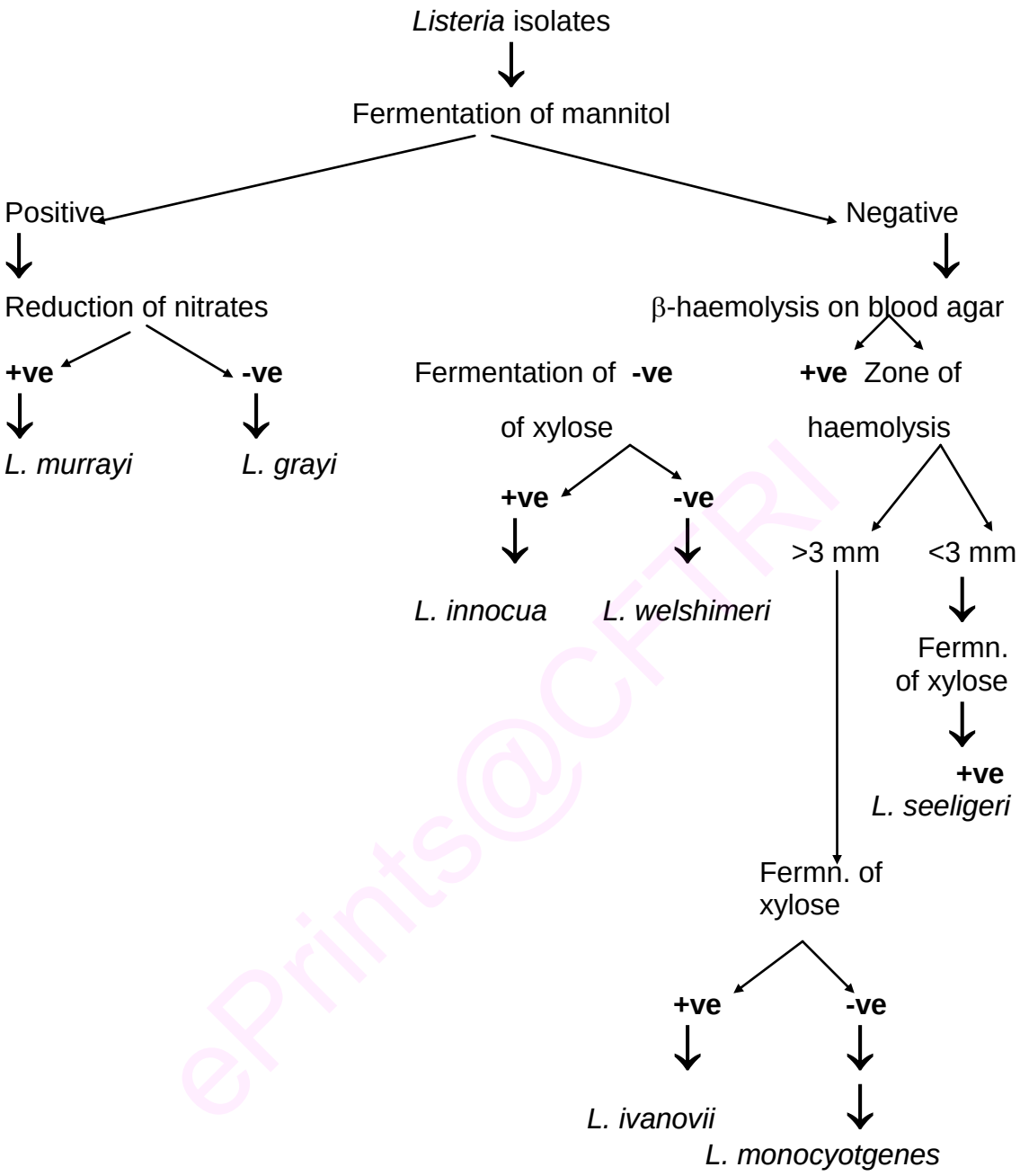
The relationship of *Listeria* to other bacteria remained obscure until 1970s. It was not documented in the first three editions of Bergey's Manual of Determinative Bacteriology. The 4th edition in 1934 included *Listeria* in the tribe Kurthia of Cornyebacteriaceae family (Bergey's Manual of Determinative Bacteriology, 4th edition, 1934).

For many years, the genus *Listeria* was monospecefic, containing only type species-*L. monocytogenes.* Later on, *L. denitrificans* was added in 1948; *L. grayi* (in honour of M. L. Gray) in 1966; *L. murrayi* (in honour of E. G. D. Murray) in 1981; *L. ivanovii* (in honour of I. Ivanov) in 1985; *L. welshimeri* (in honour of H.J. Welshimer) and *L. seeligeri* (in honour of H. P. R. Seeliger) in 1983(Bergey's Manual of Systematic Bacteriology, 1986).

**2.2.4 Characteristics**

*Listeria* is a small Gram positive rod measuring about 0.5 microns in diameter and 1-2 microns in length with rounded ends. Cells are found singly or in short chains. In older cultures, it is Gram variable and appears like diphtheroids, cocci or diplococci leading to frequent mis-identification. *Listeria* does not produce spores and capsules are not formed. Bacterium is motile with its peritrichous flagella. It shows characteristic tumbling motility, when cultured at temperatures below 30°C and non-motile at 37°C. In semisolid culture medium, it shows typical umbrella shaped motility.

On nutrient agar, colonies are 0.2-0.8 mm in diameter smooth, punctiform, bluish gray, translucent and slightly raised. It produces typical blue-green iridescence when observed under obliquely transmitted light. *Listeria* usually grows on most commonly used bacteriological media. The growth rate is increased by the presence of fermentable sugar, particularly glucose (Daneshvar et al. 1989). *Listeria* can grow in wide range of temperatures from 1-2°C to 45°C, with temperature optimum of 37°C. It grows from pH 4.4 to 9.6 with the optimum pH of 7. Growth can occur with 10% sodium chloride and survival can be there even at higher concentrations.




Key for the identification of *Listeria* spp.

*Listeria* is aerobic, microaerophilic, facultatively anerobic, catalase positive and oxidase negative. *Listeria* is homofermentative and oxidizes glycolytic intermediate compounds. All strains grow on glucose, fructose, dextrin, salicin and glycerol producing acid without gas. All strains are positive for Methyl-red and Voges-Proskauer test. Urea is not hydrolysed and indole is not produced. All *Listeria* species are phenotypically very similar. They are differentiated by hemolysis test, acid production from D-Xylose, L-rhamnose, alpha methyl-D-mannoside and mannitol.

### 2.2.5 Listeriosis

*Listeria monocytogenes* causes listeriosis in humans. Listeriosis has several clinical manifestations. Clinical illnesses caused by *Listeria* are outlined by febrile gasteroenteritis, bacteriemia, infection in pregnancy, neonatal infection, CNS infection, endocarditis and localized infections. Symptoms expressed depend on the age and immune status of the individual and mode of transmission (CIDRAP, 2006).

Infection to human can occur by oral, ocular, respiratory or urogenital routes (Pearson et al. 1990). Cases of cutaneous listeriosis, conjunctivitis and pneumonic listeriosis have been reported in persons in contact with infected animals (Hird et al. 1987; Marth et al. 1988). Study of outbreaks has established single food as the vector for listeriosis (Hird et al. 1987). Transplacental infection and transmission from the vaginal canal during birth are believed to cause perinatal and neonatal infections (Lamont et al. 1988).

In healthy human beings, infection can give rise to atypical meningitis, febrile gastroenteritis characterized by fever and watery diarrhea of 2-3 days duration,

bacteriemia characterized by acute febrile illness, often with myalgias, arthritis, headache, and backache or remain asymptomatic (Schlech et al. 2000).

Pregnant women have 17-fold increase in risk of listerial bacteriemia. Listeriosis occurs usually in the third trimester of pregnancy. Pregnant women generally experience influenza like illness with or without gastrointestinal problem (Weinberger et al. 1984; Mylonakis et al. 2002). Intrauterine infection occur via maternal bacteriemia and can lead to amnionitis, preterm labor, spontaneous abortion, still birth, or infection to neonate.(Schuchat et al. 1991). About 22% of perinatal infection result in still birth. Two thirds of surviving infants manifest neonatal listeriosis (Lorber et al. 2005).

Neonatal illness can occur as early onset or late. Early onset of the disease resulted from uterine infection can detected after birth. Infants generally present with sepsis, respiratory distress, fever and neurologic abnormalities. Death can occur from overwhelming disseminated infection (Mylonakis et al. 2002). Late onset of disease occurring two weeks after birth may be via noncosmic transmission. The disease will present as meningitis (Braden et al. 2003). In immunocompromised adults, CNS infection due to listeriosis leads to meningitis, rhombencephalitis and brain abscess. CNS infection has caused mortality of 22%. Endocarditis occurs in 7.5% of adults. Listerial infection causing vacuolar heart disease has a mortality rate of 48% (Lorber et al. 2005). Listeriosis in animals is due to *L. ivanovii*. The infection to CNS has caused the typical circling disease to the animals (Gill et al. 1937).

### 2.2.6 Pathogenesis

The pathophysiology of *Listeria* infection in humans and animals is still poorly understood. The information derived from interpretation of epidemiological, clinical and

histopathological findings and observations made on experimental infection in animals has shown the pathogenicity of *L. monocytogenes.*

The ingested *L*. *monocytogenes* colonise in the host tissues by crossing the intestinal barrier, followed by multiplication in liver. The presence of bacteria in the absorptive epithelial cells of the apical area of villi in guinea pigs by Racz et al. (1972) has evidenced the crossing of intestinal border. Electron microscopy of hepatic tissue from infected mice suggests that *L*. *monocytogenes* goes through the complete infectious cycle in hepatocytes (Gaillard, 1996). The intracellular life cycle of pathogen is by phagocytosis, lysis of the phagocytic vacuole, movement in the cytoplasmic environment and cell to cell spread. The surface protein internalin was identified as the factor responsible for invasion of the organism. The protein family internalins comprises invasion proteins InlA and InlB. Surface protein, InlA is anchored covalently to bacterial cell wall by its well-characterized LPTTG motif in C-terminal domain. Leucine rich repeats in the N-terminal and LPTTG motif in C-terminal promote the bacterial entry into target cells (Lecuit, 1997). InlA interacts with the transmembrane glycoprotein E-cadherin on the target cells and allows the bacterial entry (Schubert, 2002). *Inl*B, the soluble protein interacts with glucosaminoglycans and potentiate the receptor protein Met-mediated endocytosis thereby internalization of bacteria (Veiga, 2005).

Usually, escape from the phagocytic vacuole is mediated by Listeriolysin O a product of *hly*A gene. The disruption of phagosomal membranes was studied by earlier researchers and the mechanism was demonstrated using *hly* mutants (Armstrong, 1966; Kingdon, 1970; Gaillard; 1987). Active LLO secreted during the invasion phase potentiates the entry of *L. monocytogenes*. Once the bacteria are within a phagosome, the LLO becomes more active due to acidic pH in the intraphagosomal environment,

favouring the lysis of this compartment. After the phagosome is broken, the bacteria are freed in the cytoplasm, where the LLO is neutralized by neutral pH of the cytosol (Beauregard, 1997).

Movement within the cytoplasmic environment is due to actin-based motility. Bacterial surface protein ActA was identified as the molecular determinant for the motility (Kocks, 1992; Domann, 1992). *Act*A is a polarized protein anchored non-covalently to the cell wall by a transmembrane hydrophobic tail motif on its carboxyl terminal domain that traverses the cytoplasmic bacterial membrane (Kocks, 1992). *Act*A is responsible for the polymerization of actin enriched structures named 'actin comet tails' that enable the bacteria to move in the host cell cytoplasm. VCA region of ActA polymerizes actin filaments at one bacterial end (Boujemaa-Paterski, 2001). These filaments will push the bacteria in the intra-cytoplasmic environment, helping them to reach plasma membrane and eventually leading to the invasion of neighboring cell by a cell-to-cell spread mechanism (Monack, 2001).

The experiments conducted with gestating animal hosts have shown that *L*. *monocytogenes* gains access to foetus by hematogenous penetration of placental barrier. Experiments were carried out with sheep, cattle, rabbits and guinea pigs. Human placental infection is characterized by numerous microadscesses and fecal necrotizing villitis (Parkassh, 1998). Invasion of *L. monocytogenes* in brain was derived from the meningitis symptom in listeriosis infection. This was evidenced by the invasion of *L*. *monocytogenes* invitro in neurons (Dramsi et al. 1998).

These cascades of events of intracellular growth are the consequence of the virulence gene products. The virulence genes of *L. monocytogenes* are clustered on the

chromosome in the so called *prf*A dependent virulence gene cluster. The cluster comprises six well characterized genes *prf*A, *plc*A, *hly*, *mpl*, *act*A and *plc*B and three small open reading frames of unknown functions, downstream of *plc*B and called X, Y, and Z (Gouin et al. 1994, Lampidis et al. 1994).

### 2.2.7 Pathogenic Determinants / Virulence Factors

#### *2.2.7.1 Haemolysin*

The haemolysin gene, *hly* was the first virulence determinant with precise role in pathogenicity identified and sequenced in *Listeria spp*. The gene product Listeriolysin O is responsible for the formation of beta-hemolysis on blood agar. This strong correlation between the hemolytic activity and pathogenicity in the genus *Listeria* is well documented (Groves et al. 1977). Listeriolysin O is a 58-60 kDa sulfhydryl activated, pore forming haemolysin. LLO belongs to the family of cholesterol dependent cytolysins. Cholesterol acts as a receptor for this protein. This thiol activated toxins form discrete, oligomeric pores in the membranes containing cholesterol (Bhadki et al. 1985). This toxin is antigenically and genetically related to streptolysin O, ivanolysin O, pneumolysin O, alveolysin and seeligerolysin. The acidic pH range for its optimal activity has adapted the protein to function within the phagosomal component. In addition to the formation of discrete pores, more extensive perturbations such as disruption and fusion as well as lipid extraction from membranes have been described (Bonev et al. 2001).

#### *2.2.7.2 Act A*

ActA a product of gene *Act*A present downstream to *hly* is the protein responsible for intracytoplasmic movement and cell to cell spread. Unusual *Act*A mutant studies have revealed incapability of bacteria to spread. Proline rich regions of this 90 kDa

protein acts as a nucleator and polymerizes actin filaments to form comet tails (Kock et al. 1993).

#### *2.2.7.3 Internalins*

The surface protein internalins are encoded by *inl*A and *inl*B loci that are thermoregulated at the transcriptional level by *prf*A dependent and independent manners (Dramsi et al. 1993; Lingnau et al. 1995). Transposon mutagenesis and inframe deletions of *inl*AB locus have shown the role of internalin as invasion protein. Both the proteins *Inl*A and *Inl*B endow the bacterial entry into nonphagocytic cells (Lingnau et al. 1995).

#### *2.2.7.4 Other pathogenic determinants*

There are several other virulence factors, which contribute indirectly. They help in housekeeping and allow the organism to survive in host.

Invasion associated protein p60

P60 (*iap*) a gene and its product responsible for formation of stable rough appearance to the colony on agar plates. It has a murein hydrolase activity, which is required for normal septum formation and essential for cell viability. As p60 mutants are lethal (Wuenscher et al. 1993). This 60 kDa extracellular protein is involved in the invasion of *Listeria* spp. into fibroblat cells (Kuhn et al. 1989).

Phospholipases

Production of phospholipases in *L. monocytogenes* was first described in 1962 as opacity reaction on egg yolk agar (Fuzi, 1962). Three different enzymes with phospholipase C activity are involved in virulence. *Plc*A and *plc*B are present in *L.*

*monocytogenes* and *L*. *ivanovii,* while the third *smc*L is specific to *L*. *ivanovii*. *Plc*A, a 29 kDa protein is the product of *plc*A gene present upstream from *hly* gene. *Plc*B, a 33 kDa protein, a product of *plc*B gene is the precursor of PC-PLC. These phospholipases assist in the successful escape from the phagosome (Vazquez-Boland et al. 1992).

## 2.3 DETECTION METHODS FOR *LISTERIA* SPP.

*Listeria monocytogenes* found ubiquitously in the environment is of much concern, because of its role as foodborne pathogen. The listeriosis reduction is mainly focused on zero tolerance policies. The need for improved sensitivity and reliability of detection methods has an impact on epidemiologic investigations for the trace back, which help in measures to reduce the incidence (Donnelly, 2001).

Successful isolation depends on the ability of the method to promote the growth of small number of potentially injured cells, while at the same time minimizing the growth of non-*Listeria* background organisms. The uses of HACCP strategies to effectively eliminate organisms from food environments are predicated upon the use of sensitive, reliable methods and include enrichment methods that facilitate the recovery of *Listeria*. Detection of *L*. *monocytogenes* in food products and food processing environments is accomplished by the use of a variety of standard or rapid microbiologic procedures.

### 2.3.1 Conventional methods

Historically, it has been challenging to isolate *Listeria* from food and other samples. Very low numbers of the organism in the sample was not detected by standard conventional direct plating Method. The ability of the organism to grow under low temperatures was made use in developing protocols for isolation of this organism by prolonged period of incubation at 4°C on agar plates, till the formation of visible colonies.

This method was limiting as it was taking several weeks for isolation and does not allow the isolation of injured cells.

Selective enrichment prior to detection and plating on selective media is the approved methodology for the isolation of *Listeria* in food. Enumeration-based methods can detect single *Listeria* in 25 g of sample, as the enrichment makes the organism to detect levels of $10^4$ to $10^5$ CFU/ml. The selective agents like acriflavin and nalidixic acid rapidly outnumber the competing microflora (Welshimer, 1981). Based on the incorporation of selective agents to the enrichment media, few methods are widely recommended as reference methods. The most widely used protocols are devised by the USDA-FSIS for the detection of *Listeria* in meat and poultry products and FDA for the detection of *Listeria* in milk and milk products, fruits, vegetables and sea foods. In Europe, NGFIS protocol is being widely used. Hayes et al. (1992) compared three enrichment methods with two hundred twenty nine samples. This comparative evaluation showed around 65% *Listeria* contamination by USDA-FSIS method, 74% contamination by NGFIS method. When any two methods were used together percentage success increased between 87-91%. The reference methods for the detection of *Listeria* in all foods are FDA bacteriological and analytical method (BAM) and the International Organization of Standards (ISO) 11290 method and USDA and AOAC methods for meat and environmental samples (Gasanov et al. 2005).

All the above-mentioned plating methods are genus specific. The suspected colonies with black colour and black halo on Oxford *Listeria* medium and PALCAM medium require further identification through the biochemical tests for the species-specific identification. Besides, few commercial biochemical test kits are being in use. However, legal approval depends on the country's Food Regulation.

Using chromogenic substrates for the species differentiation is recommended by BAM. Phosphatidyl-choline specific phospolipase activity is measured using chromogenic media (Notermans et al. 1991; Coffey et al. 1996). The species are differentiated in one step plating after enrichment.

### 2.3.2 Immunological methods

A major obstacle in the detection of *L. monocytogenes* in the food matrix has been the presence of complex food constituents. Due to complex food matrix, the detection method becomes less sensitive and gives false positive results. The outbreaks are usually associated with complex foods like coleslaw, cheese, deli meat and frankfurters. Immunoassays have proved to be a good alternative detection system for use in case of complex matrix. Immunoassays are fast and relatively inexpensive and allow the accurate detection of antigens after very little sample purification (Hall et al. 1989). The assay also provides the real time information and allows for the detection of timely response of the pathogen (Meng and Doyle, 2002). Immunoassays are based on the natural affinity of antibodies towards antigen. The antigen can be a hapten, a protein or a carbohydrate on the surface of the cell.

Both, extra-cellular and cell surface listerial proteins like flagellar proteins (Farber and Speirs, 1987; Skjerve et al. 1990; Fluit et al. 1993; Kim et al. 2005), internalins (Dramsi et al. 1993; Mengaud et al. 1996), listeriolysin (Parrius et al. 1986; Mengaud et al. 1987; Mengaud et al. 1988; Barclay et al. 1989; Kreft et al. 1989; Kathariou et al. 1990; Johnson et al. 1990; Nato et al. 1991; Low et al. 1992; Darji et al. 1995; Traub et al. 1995; Darji et al. 1996), actin polymerization protein (Domann, 1992, Kock, 1993, Neibuhr, 1993), bacteriolytic invasion protein p60 (Kuhn et al. 1989; Kohler et al. 1990; Gutekunst et al.. 1992; Ruhland et al.. 1993), phospholipase C (Geoffroy et al. 1991), 66

kDa aminopeptidase (Bhunia et al. 1991; 1992), delayed type hypersensitivity protein *lma*A (Gohmann et al. 1990) and other unrecognized proteins are used as antigens. The antibodies raised against these proteins were used in immunoassays for the detection of *L. monocytogenes* and *Listeria* species. In most of the cases, heat killed whole cells have been used as antigens (Mattingly et al. 1988; Beumer and Brinkman, 1989; Comi et al. 1991; Curiale et al. 1994; Ganger et al. 2000; Sewel et al. 2003; Sibermagel et al. 2005).

The antibodies are raised by immunizing an animal with multiple antigen doses. Polyclonal, monoclonal and recombinant antibodies are used for the detection of the antigen. Polyclonal antibodies have been raised against specific proteins, may be either genus-specific or species-specific. Polyclonal antibodies have been used since several decades for the detection (Breitling and Dubel, 1999). They have been used in commercial assays like TECRA *Listeria* immunoassay (TLVIA) (Knight et al. 1996) and Assurance *Listeria* polyclonal enzyme immunoassay (Feldstine et al. 1997).

Monoclonal antibodies raised in hybridoma cell lines provide indefinite availability of single antibody. Raising monoclonal antibody begins with immunization of animal with the respective antigen. As the immune response is maximized in the B-cells, the antibodies are isolated from spleen of the animal and fused with myeloma cells to produce a hybridoma cell that has the immortality of the cancer cell and the antibody producing capacity of the B-cell. Monoconal antibodies are been used in the commercial assays like VIDAS and Lister test (Allerberger et al. 2003; Sewell et al. 2003).

Recombinant antibodies are being used to cut off the antibody production cost. Recombinant antibodies are made either by expressing each chain separately and

recombined to form the Fab fragment of the antibody, which has the comparable affinities to monoclonal antibodies (Churchill et al. 2002; Joosten et al. 2003; Little et al. 2000; Yau et al. 2003) or joining the heavy and light chain by short synthetic peptide linker to form a single chain variable fragment-ScFv (Paoli et al. 2004) or different type of recombinant fragments are constructed (known as VHH), which substantiates the VH domain of the conventional antibody (Vu et al. 1997).

Antibodies are successfully utilized for the detection, quantification and concentration of the pathogen. Several methods utilize labelled antibodies as signal generation reagents (Feng, 1996), as absorbance (Wyatt, 1993), in epifluorescence microscopy (Tortorello and Gendel, 1993), fluorescence (Tu, 2001), amperometry (Hadas, 1992; Brewster and Mazenko, 1998), and electro-chemiluminescence (Yu and Bruno, 1996; Crawford, 2000). Immuno beads are very widely used (Skjerve and Olsvik, 1991; Luk and Lindberg, 1991; Fratamico et al. 1992; Gehring et al. 1996) and related approaches such as immunoaffinity columns (Brewster et al. 2003) and immunofiltration membranes (Paffard et al. 1996; Gehring et al. 1998) have been used for the detection of *Listeria.*



#### *2.3.2.1 Immunoprecipitation and agglutination assays*

Immunoprecipitation method utilizes the antibody-coated latex beads, magnetic beads or agarose beads for qualitative detection of the pathogen. Antibody-coated beads recognize specific antigen of the organism. The concentrated organism can be detected and quantified by conventional or molecular methods. Immunoprecipation method has been used to detect *L. monocytogenes* from food samples to the level of 11CFU/ml after enrichment (Feldstine et al. 1997).

Latex agglutination method makes use of antibody-coated latex beads for the specific detection of antigen. Each antigen bind to more than one antibody coated latex beads and agglutinates to show the presence of the pathogen. This qualitative method is used to detect *L. monocytogenes* by coating the beads with anti-LLO antibodies (Marter et al. 1997).

#### *2.3.2.2 Enzyme Linked Immunosorbent Assay [ELISA]*

ELISA is the most common method used in immuno-detection of pathogens, both qualitatively and quantitatively. ELISA methodology is used to detect the pathogen in complex sample matrices. Number of ELISA formats has been used, including direct ELISA, Sandwich ELISA and competitive ELISA for the identification and differentiation of *L. monocytogenes* and other *Listeria* species in food and environmental samples. In Direct ELISA, the test sample is directly coated to the microtitre plates and identified through primary antibody, specific to the protein of respective species and visualized by the chromogenic substrate which acts with the enzyme linked secondary antibody recognizing the primary antibody. The measurement of colour intensity quantifies the amount of pathogen present in the sample. In Sandwich ELISA, the plate is coated with antibody specific to the pathogen and then the sample, followed by the secondary antibody and is used to concentrate the sample, so as to increase the intensity of the signal and sensitivity of detection (Hess et al. 1998). Flagellar monoclonal antibodies are used in sandwich ELISA to detect *Listeria* to the level of $10^5$ CFU/ml (Farber et al. 1988; Kim et al. 2005). Commercial kits like TECRA *Listeria* visual immunoassay (Knight et al. 1996), Assurance India polyclonal enzyme immunoassay (Feldstine et al. 1997) have used sandwich ELISA for the genus specific detection of *L. monocytogenes*.

In Competitive ELISA, the competition between the known antigen and unknown antigen takes place, which silences the background signalling (Hess et al. 1998). *Inl*B polyclonal antibody is used to detect *L. monocytogenes* through competitive ELISA (Leonard et al. 2005).

#### *2.3.2.3 Enzyme linked fluorescence assay [ELFA]*

ELFA utilizes the fluorescence tagged antibodies for the detection of antigen. The fluorescence labeling decreases the time required for the detection by eliminating the need for colorimetric assay in ELISA. Monoclonal antibody tagged to fluorescent probe is used in VIDAS test kit for the detection of *L. monocytogenes* (Sewell et al. 2003).

### 2.3.3 Molecular methods

A major aspect associated with detection of *L. monocytogenes* is the occurrence of this organism in low numbers at which the bacteria are normally found in contaminated food samples (Hoffman and Weidman, 2001). In one outbreak, listeriosis was attributed to contamination level of < 0.3 CFU/ml of hot dog (Donnelly, 2001). The zero tolerance policy for the *L. monocytogenes* levels has necessitated the food producers to detect it to the lowest level quickly and accurately (Mc Lauchlin, 2004). The conventional enrichment based methods are time consuming and genus specific. It requires quite a longer period to confirm the species by biochemical characterization (Wan et al. 2003). Molecular methods targeting the nucleic acids for the identification of *Listeria* to its species level is the promising method towards accurate detection of low level contamination (Churchill et al. 2005).

### *2.3.3.1 Nucleic acid based probes: DNA hybridization*

Nucleic acid probes are the viable tool for the detection of *Listeria* since 1987. Target sequences in ribosomal RNA, mitochondrial DNA, chromosomal DNA or plasmid DNA is detected using the oligonucleotide probe of complimentary sequence with a label for detection. Recently, the radioactive probes are replaced by biotinylated probes, probes incorporating digoxygenin and florescence markers.

DNA hybridization tests aim towards differentiation of *L. monocytogenes* by targeting probes to virulence factor genes. The distinct signature of a bacterium 16S rRNA is also used for the species differentiation. The molecular targets used for the identification of *Listeria* by DNA hybridization include Delayed type hypersensitivity(*dth*), rRNA genes, hemolysin (*hly*), Invasion associated protein (*iap*), Putative transcriptional activator (*prf*A), Internalin A (*inl*A), Internalin B (*inl*B), Internalin C (*inl*C), internalin related protein (*irp*A), Phospholipase A and B (*plc*A, *plc*B), Clp ATpase (*clp*E), Metalloprotease (*mpl*), Actin polymerizing protein (*act*A) and Flagellin (*fla*) (Gasanov et al. 2005).

Commercially available DNA hybridization tests are routinely used for the testing of foods and have been extensively trailed for their sensitivity and accuracy (Feng, 2001). Accuprobe kit from Gen-Probe USA, Genetrak DNA hybridization kit from Neogen Corporation and VIT kit from Germany are widely used. Genetrak kit is recommended by Bacteriological analytical manual. Acuprobe is based on the hybridization of labeled DNA probes to virulence factor mRNA, thereby ensuring the detection of viable cells. Genetrak is based on the hybridization of labeled probe to 16S rRNA. VIT kit utilizes the vermicon identification technology and is based on in situ hybridization of fluorescently labeled probes to intracellular target RNA.

As an alternative to using high copy number target sequences, nucleic acid based amplification methods employing the PCR and NASBA have been reported.

### *2.3.3.2 Polymerase Chain Reaction [PCR]*

The exponential amplification of the primer defined flanking region, the PCR has become the popular tool for the detection of *Listeria* species. The presence of *L. monocytogenes* both live and dead can be detected by simply determining the presence of band on agarose gel representing the gene of interest and hence the organism.

Research carried out till now indicates PCR is more sensitive than the culture based methods for the pathogen detection in food (Aznar and Alarcion, 2002). PCR is specific because of its unique primer regions in the virulence gene. *hly*A has been used as a target gene for the detection of *L*. *monocytogenes* by Norton et al. (2001) and Thimothe et al. (2004) in smoked salmon. *iap* gene was used in detection of *Listeria* spp. in meat by Barbuddhe et al. (2002), *inl*B has been used in PCR for detection in frankfurters (Jung et al. 2003). 16S rRNA was used by Wang et al. (1992) for detection of *L*. *monocytogenes* in artificially contaminated food. Sallen et al. (1996) made a comparative analysis of 16S rRNA and 23S rRNA.

Conventional PCR is the basis for many nucleic acid based detection systems. This method involves the total DNA extraction from food sample. Two oligonucleotide primers are designed to select the specific region in the pathogen specific gene. Taq DNA polymerase exponentially amplifies the template in the presence of dNTPs to give a visible band under UV light when run on agarose gel. PCR has been used to detect *Listeria* in pure cultures (Jaradat et al. 2001; Jung et al. 2003), salmon (Wan et al. 2003), smoked salmon (Norton et al. 2001), milk and milk products (D' Agostino et al. 2004).

Detection using PCR is carried after enrichment for 16 h. Direct detection without prior enrichment has shown variable results (Bohnert et al. 1992).

Multiplex PCR, where multiple primer sets are used, allows the simultaneous detection of more than one pathogen in the same sample, such as *Listeria* and *Salmonella* (Hsih et al. 2001; Li et al. 2003) or *L. monocytogenes* and other *Listeria* species (Bubert et al. 1992; Lawrence et al.1994; Bansal et al. 1996; Wesley et al. 2002). This PCR reduces the labor cost and testing time. *Listeria* species was detected in different samples like poultry processing environment, turkey samples and pure cultures.

Nested PCR, where multiple sets of primers used in sequential reactions are designed against the same target is used to increase the sensitivity and specificity of the reaction. This method is used in detection of *L. monocytogenes* in clinical samples (Jaton et al. 1992; Olcen et al. 1995; Cocolin et al. 1997; Backman et al. 1999; Chen et al. 2000), environmental samples (Bsat et al. 1993) and milk samples (Neiderhauser et al. 1994; Herman et al. 1995; Ha et al. 2002).

In competitive PCR, competitor fragment of DNA that matches with the gene of interest is introduced into the sample. The competitor fragment is distinguished from the pathogen gene fragment by its smaller size. The PCR is performed and the pathogen gene signal is compared to that of competitor DNA on agarose gel. This method is used to identify and quantify *L. monocytogenes* in pure cultures (Schleiss et al. 2003) and milk (Choi and Hong, 2003) using *hly* gene.

One of the rate limiting steps of PCR is the analysis of amplified products on agarose gels. To overcome this problem, fluorescent resonance energy transfer (FRET) based PCR was developed. This method involves direct measurement of the fluorescence signal in the PCR product. This method was used to detect *L. monocytogenes* in milk using *hly* gene (Koo and Jaykus, 2003).

Real Time PCR is another way to eliminate the need for agarose gel electrophoresis. This method uses the SYBR Green I florescent dye to follow the PCR amplification. The increase in fluorescence after each successive cycle allows the direct quantification of DNA. This method has been used to identify and quantify *L. monocytogenes* in pure cultures, cabbage and other samples (Hein et al. 2001; Hough et al. 2002; Bhagwat et al. 2003; Rudi et al. 2003; Huijsdens et al. 2003; Rodriguez-lazaro et al. 2004). Simultaneous detection of different genes of *Salmonella* and *Listeria* (Jothikumar et al. 2003; Wang et al. 2004) was achieved by this method. Primers were designed to detect *L. monocytogenes* by *hly* gene and 23S rRNA to differentiate the species (Rodriguez et al. 2004).



#### *2.3.3.4 Reverse Transcriptase PCR*

All the above methods detect both the live and dead bacteria as DNA lasts for several years. To detect only the live pathogen is a requirement for the food processors. Detection of pathogen by using RNA rather than DNA is the indication of live cells. RT–PCR makes use of reverse transcriptase enzyme, which in the presence of complimentary primer creates cDNA from RNA strand corresponding to the transcribed gene. The cDNA is then amplified using the oliginucleoide primers and DNA polymerase under normal PCR conditions. This two-step method has been used to detect *L. monocytogenes* in artificially inoculated meat samples by targeting mRNA transcripts of

*hly, prf*A and *iap* genes (Klein et al. 1997), in waste samples by targeting transcripts of 23S rRNA genes (Burtscher et al. 2003) and heat injured *Listeria* by *hly* transcript (Koo and Jaykus, 2003).

### *2.3.3.5 Nucleic acid sequence based amplification (NASBA)*

NASBA is a non-automated enzyme based reaction, which helps in the detection of pathogen. This method utilizes RNA for the detection of viable bacteria. The total RNA extracted from the sample acts as the template. First the reverse transcriptase used in combination with oligonucleotide primer produces a cDNA-RNA hybrid. RNase enzyme removes the RNA from the hybrid molecule and allows the reverse transcriptase to synthesize a double stranded cDNA. cDNA generates RNA transcripts in the presence of T7 polymerase. The single stranded RNA produced is visualized by agarose gel electrophoresis or by using specific oligonucleotide probes for hybridization assays combined with colorimetric detection. This method has been used to detect viable *L. monocytogenes* using 16S rRNA sequences and *hly* mRNA in food (Uyttendaele et al. 1995; Blais et al. 1997; Beumer and Hazeler, 2003).

### *2.3.3.6 DNA microarray*

DNA Microarray is the existing newer technology based on DNA or RNA hybridization. This method is used to investigate microbial evaluation and epidemiology and can serve as a diagnostic tool for clinical, environmental or food testing. Microarrays are composed of discretly located DNA probes fixed on to the solid support such as glass or membrane. The probes are specific to either specific oligonuceotides or PCR products.

In PCR based microarrays, PCR is performed using universal primers to amplify all possible genes of interest. The purified PCR products are labelled with fluorochromes and allowed to bind to the probes in the array. In oligonucleotide-based microarrays, total RNA from the bacteria is directly labelled with fluorocromes prior to hybridization.

PCR-based method has been used to detect *Listeria* in environmental samples using 16S rRNA sequences (Call et al. 2003). Multiple primer sets have been used in PCR and the products are used in microarrays and the non-resolvable bands in agarose gels are detected (Volokhov et al. 2002). Microarrays have also been used to detect different *Listeria* spp. and *Campylobacter* spp. of clinical relevance to the level of 200 CFU/ml (Lampel et al. 2000; Sergeev et al. 2004). Phylogenetic relationship of *L. monocytogenes* serotypes has also been described by this method (Call et al. 2003; Zhang et al. 2003; Doumith et al. 2004).

***2.3.3.7 Immuno PCR***

The major obstacle in the use of PCR in food and environmental samples is the presence of inhibitors, which gives false positive results (Bickley et al. 1994). Several steps have been made to remove the inhibitory factors by sample treatment (Lantz et al.. 1996). Magnetic beads, dip-sticks and membranes are used to remove target DNA from reaction inhibiting sample matrices (Lampel et al. 2000; Li et al. 2000; O'connor et al. 2000; Hsih et al. 2001; Hudson et al. 2001; Garrec et al. 2003). Antibodies have been used successfully to capture the pathogen by immunomagnetic beads (Skjerve and Olsvik, 1991; Luk and Lindberg, 1991; Fratamico, 1992; Gehring, 1996) and immuno-sepharose beads (Gray and Bhunia, 2004). The concentrated pathogens were used as template for PCR. Immuno PCR has been used to detect *L. monocytogenes* in cheese (Fluit et al. 1993), ham samples (Hudson et al. 2001) and raw milk.

## 2.4 VIRULENCE DETERMINATION

Pathogenicity within the genus *Listeria* is restricted to two species, *L. monocytogenes* and *L. ivanovii*. Several models, both in vivo and in vitro have been used to demonstrate the pathogenic potential of these organisms. The best model still used to determine virulence is CAMP test, mouse pathogenicity test, anton test and inoculation of chorioallantois membrane of chicken embryos. FDA employs the mouse virulence test to demonstrate conclusively the virulence potential of isolates.

### 2.4.1 CAMP test

The ability of synergistic lysis of RBC by *L. monocytogenes* was first observed by Fraser in 1962. Studies showed enhanced haemolysis in sheep blood or rabbit with *Staphylococcus aureus* or *Rhodococcus equi* by *Listeria* spp (Fraser et al. 1964). The development of CAMP test has been described by Christie et al. (1944). When haemolytic *streptococci* were inoculated onto the surface of sheep blood agar, enhanced lysis was observed adjacent to colonies of beta toxin producing *S. aureus*. This synergistic phenomenon was used in virulence determination of *Listeria* species along with *R. equi* (Smola et al. 1989). The virulence in *Listeria* spp restricted to the two spp. was differentiated using this method (Groves and Welshimer, 1977; Gaillard, 1986; Nato et al. 1991; Portnoy et al. 1992; Neibuhr et al. 1993; Sanchez et al. 1995).

### 2.4.2 Mouse virulence test

Mouse virulence assay provides an *in vivo* measurement of all virulence determinants and is often used as a reference standard for *L. monocytogenes* virulence (Mackaness et al. 1962). This assay is carried out by inoculating groups of mice with various doses of *L. monocytogenes* via oral, nostril, intraperitoneal, intravenous or subcutaneous route. The virulence of the given bacteria is then determined by the

mouse mortality The infective dose was estimated by corresponding colony forming units of the bacteria through plate counts and is commonly expressed as medium lethal dose ($LD_{50}$) (Reed et al. 1938; Welkos et al. 1994).

### 2.4.3 Mutation studies

Several mutation studies have been carried out to determine the virulence of *Listeria*. The mutations in the virulence genes have proven the avirulence of the organism. Tn916-induced mutation in the hemolysin has shown to affect the virulence of *Listeria* (Kathariou et al. 1987). A novel mutation in the central *L. monocytogenes* regulator, *Prf*A which resulted in defective intracellular growth and cell to cell spread, has proved to be nonpathogenic (Sun et al. 1990). Transposon mutation within *plc*A gene has proved the avirulence of *L. monocytogenes* (Camilli et al. 1993). Mutations in P60, an extracellular protein have shown the avirulence of *Listeria* by not invading professional phagocytes (Khun et al. 1989).

### 2.4.4 Cell culture models

The *in vitro* cell culture models have played an important role in *L. monocytogenes* virulence. The invasion assays carried out by different species have provided the complete picture of *Listeria* virulence. Epithelial like cell lines, caco-2 was used for the determination of pathogenesis. Two cell culture assays, the PM assay, where virulence is assessed through the penetration and multiplication within the cell and PF assay, in which case, virulence is assessed through the plaque forming ability of *L. monocytogenes* strains were used in caco-2 cell lines (Van Langendonck et al. 1998). Macrophage-like cell lines and human erythrocytes were also used as the models to determine the virulence. Recently, *Drosophila* S2 cell lines have been considered as the best model to study virulence of *Listeria* (Cheng et al. 2003).

## 2.5 INCIDENCE IN FOODS

*Listeria* being ubiquitous in nature is a common contaminant in food. The listeral incidence is well documented in milk and dairy products, meat and poultry, fish and sea food, and vegetables. It can enter as a secondary contaminant in the processing plants.

The 1983 listeriosis outbreak in Massachusetts provided the evidence for the presence of *Listeria* in milk. Surveillance by Center for Disease Control and Prevention and other organsations have shown 2-33% of raw milk contamination with *Listeria*. The survey in different parts of world have shown the presence of the organism in milk products like cheese, ice-creams, pasteurized milk, butter, sweetened condensed milk and dry milk powder (Ryser, 1999).

USDA-FSIS monitoring programme has shown a wide range of raw meat, meat products like jerky, sausages, roasted, cooked or cornered beef, hot dogs, hams contaminated with *Listeria*. Few of these products like ham and hotdogs have caused major outbreaks. Around 7% of raw meat and 2-24% of meat products contains *Listeria* (Farber and Peterkin, 1991). Sea foods are the potential source of *Listeria.* The aquatic creatures like fish, oysters, shrimps, crabs, lobsters containing *Listeria* have been recorded. FDA has shown 8.7 -24.3% of sea food contamination.

Vegetables like lettuce, cabbage, carrots, cucumber, and potatoes have been documented as the source of *Listeria* in vegetables. The product coleslaw prepared from cabbage was the source of an outbreak in Canada 1981. The salads tested for *Listeria* harboured 2-15% of *Listeria*.

In processing plants *Listeria* is present as a surface contaminant. Sampling of nearly 346 processing plants revealed, that 35.3% contained one or more *Listeria* (Ryser and Marth, 1996).

In the present scenario of globalization and the increasing use of minimally processed and ready-to-eat foods, there appears a need to study characterization of hazards associated with *Listeria* spp. and the relevant risk analysis of *Listeria* spp and listeriosis. There also arises a need to characterize the native listerial isolates occurring in diversified habitats by various techniques of specificity and reliability like those of immunological and molecular, besides those of conventional approaches, which have stood the test of time. Primarily, all these developments should find ample applications to detect *L*. *monocytogenes* and closely related pathogenic species of Listeria in foods.

One of the emerging disciplines of food safety has been the “Foods Safety Risk Analysis”. Risk assessment is one of the method by which science can be used to address food safety. Risk assessment is a part of risk analysis and the other two components are risk management and risk communication. The risk analysis includes recommendations for Good Manufacturing and Distribution Practices (GMDPs) and Good Hygienic Practices (GHPs). This may result in criteria for microbiological hazards in foods.

## 3.0 OBJECT AND SCOPE OF THE STUDY

*Listeria monocytogenes* has been a bacterial foodborne pathogen of serious health concern, which is responsible for listeriosis, an illness characterized by meningitis, encephalitis and septicaemia leading to bacteriaemia and still birth in pregnant women and cutaneus lesions and flu-like symptoms in healthy persons. The mortality rate for those contracting listeriosis is approximately 20%. Besides, *L*. *monocytogenes*, *L. ivanovii* and *L. seeligeri* are also known to cause illnesses in animals. The presence of non-pathogenic *Listeria* spp. like *L. grayi* and *L. innocua* indicates the potential for the occurrence of pathogenic species, *L*. *monocytogenes*. *Listeria* being ubiquitous in nature, is a common contaminant in foods. In view of this, listeriosis outbreaks have been reported through consumption of a diverse range of foods, irrespective of product profile and food constituents. The listeral incidence is well documented primarily in milk and dairy products, meat and poultry, fish and seafoods and vegetables. Further, this organism does occur as a secondary contaminant in foods.

It is essential to detect the pathogen in low numbers in food samples in the shortest possible time with a high degree of specificity. Conventional testing methods developed for the detection and enumeration of *L. monocytogenes* from foods, using specific culture media, followed by a series of tests for confirmation are standard methods. However, these methods are cumbersome and time-consuming and often lead to under estimating the actual populations present in foods. To overcome these limitations, molecular and immunological techniques are being applied for the rapid and specific detection of *L. monocytogenes* and other *Listeria* spp. Detection methods based on antibodies (ELISA) and molecular techniques (PCR) are sensitive and the testing could be completed within a day.

In the light of prevailing Indian scenario and the global focus on *L. monocytogenes* and other *Listeria* spp. as major issues of concern in microbial food safety, the present Ph.D. programme attempts to assess the prevalence of this pathogenic species in selected types of foods being marketed locally using the conventional protocols. Further, the native isolates were assessed for their virulence factors through the development and optimization of immunological and molecular methods (ELISA and PCR) for the detection of *L. monocytogenes* in culture broth system and food matrix such as milk samples.

The objectives of the Ph.D. programme were as follows:

- Assessing the magnitude of incidence of *Listeria monocytogenes* and other *Listeria* spp. in foods
- Immunological and molecular methods for specific detection of *Listeria* spp. in pure culture and food systems
- Assessing virulence factors of *Listeria* spp.

## 4.1 PREVALENCE OF *LISTERIA* SPECIES IN DIVERSIFIED FOODS

In the global scenario, microbial food safety is viewed with high public health concern. In an estimate of foodborne illness occurring, world-wide, it has been reported to the extent of 75%. Improper reporting system in most of the countries has failed to reflect the true magnitude of incidences. It is estimated that the underreporting is as much as to the extent of 95-99%. Majority of the diseases caused by enteric pathogens are transmitted through food and water. It is impossible to have a risk-free food chain system. Food habits are highly diversified, which gives ample opportunities for the entry and proliferation of undesirable foodborne pathogenic bacterial species. Among the several foodborne bacterial pathogens of significance, strains of *Listeria monocytogenes* and other related species of *Listeria* are of importance.

It has been a challenging task to isolate *Listeria* spp. from food and related samples as it has wide ranging survival ability under extremes of nutritional and environmental conditions like very low temperatures, high salt concentrations and low pH levels. Identification of the organism by conventional culture dependent enrichment based plating methods is still considered as the golden standard. The recommended standard methods of FDA and USDA are used as reference methods for the detection of *L. monocytogenes* in food samples. Considering India as a major country in the expanded food trade business, there appears to be a need to assess a variety of commercially viable food products for *L*. *monocytogenes* and other *Listeria* species.

This experimental Chapter attempts to assess the prevalence of *Listeria* spp. including *L. monocytogenes* in milk and milk-based foods, meat and meat-based products, cereal and legume-based foods and vegetables by selective enrichment and plating protocols, followed by identification of the native isolates by morphological, cultural and biochemical characteristics.

### 4.1.1 MATERIALS

All glasswares, media and other materials used in the present study were either wet sterilized or dry sterilized. Wet sterilization was carried out at 121°C for 20 min in an autoclave and dry sterilization at 180°C for 4 h in a Hot Air Oven. All bacteriological media used were those of dehydrated media procured from Hi-Media Lab., Mumbai, India (Hi-Media, 2003). The media were prepared as per manufacturer's instructions. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

#### 4.1.1.1 Food samples

The samples consisted of the following, which were collected locally in the City of Mysore, Karnataka State, India:

i) Milk and milk products

Fifty (50) samples of raw whole milk

Fifteen (15) samples of milk products [Ice cream, Milk powder, Butter, Ghee (clarified butter), traditional milk sweets like Peda, Milk sandwich]

ii) Meat and meat products

Forty (40) samples of raw meat of chicken, mutton and fish

Ten (10) samples of processed products like chicken puff, chicken roll and chicken burger

iii) Cereal and legume-based foods

Ten (10) samples of traditional fast foods like pani puri, masala puri, bhel puri, and samosa

iv) Vegetables

Fifty five (55) samples of raw vegetables like beans, beet root, cabbage, carrot, cauliflower, chou chou, coriander leaves, mint leaves, onion, potato, pumpkin, raddish, spinach and tomato

All the samples were collected under sterile conditions by avoiding any external contamination in appropriate pre-sterilized containers (like screw-capped tubes, sterile polypropylene pouches and glass beakers and/or conical flasks). The collected samples were placed in an ice-box, brought to the laboratory for analysis within 30 min of collection and immediately subjected to analysis.

**4.1.1.2 Reference cultures**

These included strains of (i) *L. monocytogenes* Scott A, *L*. *monocytogenes* V7 and *L. innocua* Prd 01 obtained through the courtesy of Dr. A.K. Bhunia, Purdue University, USA and (ii) cultures of *L. ivanovii* Mng 01, *L. seeligeri* Mng 02, *L*. *grayi* subsp. *grayi* Mng 03 and *L*. *grayi* subsp. *murrayi* Mng 04 obtained through the courtesy of Dr. I. Karunasagar, College of Fisheries, KVAFS University, Mangalore, India. The individual cultures were maintained at 4°C on brain heart infusion (BHI) agar slants and subcultured at 15 d intervals. The cultures were propagated twice in BHI broth at 37°C prior to use in experiment.

**4.1.1.3 Diluent**

The diluent used was 0.85% normal saline, which was dispensed in requisite quantities in suitable glass containers and autoclaved.

**4.1.1.4 Gram stain** (Acuff, 1992)

Crystal violet

| | |
|---|---|
| Crystal violet | 2.0 g |
| Ethyl alcohol (95%) | 20.0 ml |
| Ammonium oxalate | 0.8 g |
| Distilled water | 80.0 ml |

Crystal violet and ammonium oxalate were dissolved in ethyl alcohol and distilled water, respectively and the two solutions were then mixed. The prepared stain was filtered and stored in a clean and dry glass stoppered bottle.

Lugol's iodine (mordant)

| | |
|---|---|
| Iodine | 1.0 g |
| Potassium iodide | 2.0 g |
| Distilled water | 300.0 ml |

Safranin (counter stain)

| | |
|---|---|
| Safranin-0 | 2.5 g |
| Ethyl alcohol (95%) | 100.0 ml |

Ten ml of the above stock solution was mixed with 90 ml of distilled water for use as counter stain.

**4.1.1.5 Bacteriological media**

*4.1.1.5.1 Blood agar*

To 100 ml of molten and tempered sterile blood agar base medium, 5% of freshly prepared defibrinated sheep blood was added, mixed well and poured in sterile petri plates.

*4.1.1.5.2* (A) *Brain heart infusion (BHI) broth and agar*

The medium composition is as follows (g/l):

| | |
|---|---|
| Peptic digest of animal tissue | 10.0 |
| Calf brain, infusion | 12.5 |
| Beef heart infusion | 5.0 |
| Dextrose | 2.0 |
| Sodium chloride | 5.0 |
| Disodium phosphate | 2.5 |
| Final pH | $7.2 \pm 0.2$ |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate quantities into Erlenmeyer conical flasks and/or test tubes (18 x 150 mm) plugged with cotton and autoclaved.

BHI agar medium was prepared by using agar at a strength of 1.5% in BHI broth medium. The agar medium was boiled to dissolve the agar, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10 ml amounts in test tubes (18 x 150 mm) plugged with cotton and autoclaved. After autoclaving, tubes containing BHI agar were kept in a slanting position, so as to have 1" butt and remaining slants.

*4.1.1.5.2* (B) *Brain heart infusion – egg yolk agar*

To the requisite quantity of melted and cooled (tempered to 50°C) BHI agar medium, 5% egg yolk emulsion (prepared under aseptic condition) was added, mixed well and poured into pre-sterilized petri plates. Poured plates were allowed to reain for 30 min at ambient temperature for solidification of medium. Poured plates were used in the experimental trials within 24 h of preparation.

*4.1.1.5.3 Hugh Leifson medium*

The medium composition is as follows (g/l):

| | |
|---|---|
| Peptone | 2.0 |
| Sodium chloride | 5.0 |
| Glucose | 10.0 |
| Dipotassium phosphate | 0.3 |
| Bromo thymol blue | 0.05 |
| Agar | 2.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 3 ml amounts in the test tubes (12 x 75 mm) and autoclaved.

*4.1.1.5.4 Kohn Two tube medium No. 1*

The medium composition is as follows (g/l):

| | |
|---|---|
| Beef extract | 2.0 |
| Peptone | 15.0 |
| Yeast extract | 2.0 |
| Dextrose | 1.0 |
| Mannitol | 10.0 |
| Phenol red | 0.05 |
| Agar | 15.0 |
| Final pH | 7.2 ± 0.2 |

Prior to use, to the molten and tempered sterile medium, add 25 ml of 40% membrane filtered urea solution, mix well and prepare 1” butt and slants using sterile glass test tubes.

*4.1.1.5.5 Listeria enrichment broth - LEB (modified)*

The medium composition is as follows (g/l):

| | |
|---|---|
| Tryptose | 10.0 |
| Yeast extract | 5.0 |
| Beef extract | 5.0 |
| Sodium chloride | 20.0 |
| Disodium phosphate | 9.6 |
| Monopotassium phosphate | 1.36 |
| Esculin | 1.0 |
| Nalidixic acid | 0.02 |
| Acriflavine HCl | 0.012 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in suitable glasswares and autoclaved.

*4.1.1.5.6 Listeria Oxford medium base*

The medium composition is as follows (g/l):

| | |
|---|---|
| Peptone special | 23.0 |
| Lithium chloride | 15.0 |
| Sodium chloride | 5.0 |

| | |
|---|---|
| Corn starch | 1.0 |
| Esculin | 1.0 |
| Ferric ammonium citrate | 0.5 |
| Agar | 10.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in Erlenmeyer conical flasks and autoclaved.

*4.1.1.5.7 Listeria Moxalactam Supplement*

This is an antibiotic supplement and is an essential requirement for the use of Listeria Oxford Medium in the isolation protocols. The ready-to-use vial with 20 mg content was reconstituted in 5 ml sterile Milli Q water, gently agitated for complete mixing of content in water and then mixed with sterile molten Listeria Oxford medium tempered to 45°C. Pre-poured plates of this selective medium were prepared by pouring approximately 20 ml aliquots of medium per sterile petri plate and allowed to solidify at ambient temperature and used within 24 h of pouring the medium.

*4.1.1.5.8 MacConkey agar*

The medium composition is as follows (g/l):

| | |
|---|---|
| Pancreatic digest of gelatin | 17.0 |
| Peptic digest of animal tissue | 1.5 |
| Casein enzymic hydrolysate | 1.5 |
| Lactose | 10.0 |
| Bile salts | 1.5 |
| Sodium chloride | 5.0 |
| Neutral red | 0.03 |
| Crystal violet | 0.001 |
| Agar | 15.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in Erlenmeyer Conical flasks and autoclaved.

*4.1.1.5.9 MR-VP medium -Buffered glucose broth*

The medium composition is as follows (g/l):

| | |
|---|---|
| Buffered peptone | 7.0 |
| Dextrose | 5.0 |
| Dipotassium phosphate | 5.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 10 ml amounts in the test tubes (18 x 150 mm) and autoclaved.

*4.1.1.5.10 Nitrate broth*

The medium composition is as follows (g/l):

| | |
|---|---|
| Beef extract | 3.0 |
| Peptone | 5.0 |
| Potassium nitrate | 1.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 5 ml amounts in the test tubes (18 x 150 mm) and autoclaved.

*4.1.1.5.11 Nutrient broth and agar*

The medium composition is as follows (g/l):

| | |
|---|---|
| Peptic digest of animal tissue | 5.0 |
| Beef extract | 1.5 |
| Yeast extract | 1.5 |
| Sodium chloride | 5.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in Erlenmeyer conical flasks and /or test tubes (18 x 150 mm) and autoclaved.

Nutrient agar medium was prepared by using agar at a strength of 1.5% in nutrient broth medium. The agar medium was boiled to dissolve the agar, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10 ml amounts in test tubes (18 x 150 mm) plugged with cotton and autoclaved. After autoclaving, tubes containing nutrient agar were kept in a slanting position, so as to have 1” butt and remaining slants.

*4.1.1.5.12 Plate count agar*

The medium composition is as follows (g/l):

| | |
|---|---|
| Casein enzymic hydrolysate | 5.0 |
| Yeast extract | 2.5 |
| Dextrose | 1.0 |
| Agar | 12.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in Erlenmeyer conical flasks and autoclaved.

*4.1.1.5.13 Tryptone broth*

The medium composition is as follows (g/l):

| | |
|---|---|
| Tryptone | 10.0 |
| Sodium chloride | 5.0 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 5 ml amounts in the test tubes (18 x 150 mm) and autoclaved.

*4.1.1.5.14 Sugar solutions*

Requisite quantities of the stock solutions (10% each) of the following sugars were individually prepared in Milli Q water, membrane filtered (0.22 mμ filters) and stored in sterile screw-capped tubes at -20°C. The sugars used were mannitol, rhamnose and xylose.

*4.1.1.5.15 sugar fermentation basal medium*

The medium composition is as follows (g/l):

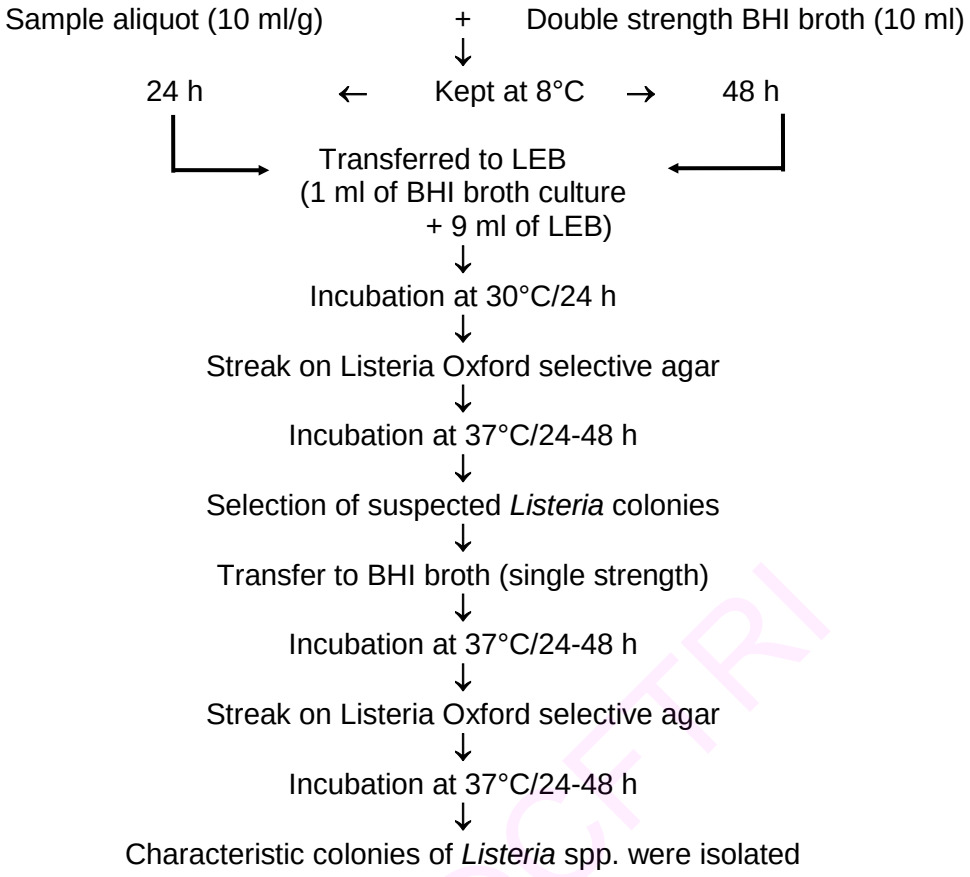
| | |
|---|---|
| Peptone | 10.0 |
| Sodium chloride | 5.0 |
| Beef extract | 3.0 |
| Bromo cresol purple | 0.04 |
| Final pH | 7.2 ± 0.2 |

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 3 ml amounts in the test tubes (15 x 125 mm) and autoclaved.

**4.1.2 METHODOLOGY**

**4.1.2.1 Isolation of native food isolates of *Listeria* species**

The non-selective cold enrichment method used by Kamat and Nair (1994) for the isolation of *Listeria* spp. from fish and meat was followed in the present study with an additional intermediate step of non-selective enrichment and subsequent plating on the selective agar. The protocol is represented schematically as follows:




In case of raw whole milk and ice cream (melted), individual samples in 10 ml aliquots were added aseptically to 10 ml quantities of double strength BHI broth, mixed well and kept at 8°C. Similarly, in case of all other samples, 10 g quantities of individual samples were added aseptically to 10 ml quantities of double strength BHI broth, mixed well and kept at 8°C. One-ml aliquots of the samples were drawn at 24 and 48 h, respectively, added to 9 ml amounts of LEB, mixed well and incubated for 24 h at 30°C. One loopful of this incubated culture broth was streaked onto Listeria Oxford selective agar and incubated at 37°C for 24-48 h. Suspected individual colonies of *Listeria* with the characteristic colony morphology [isolates of *Listeria* spp. form shiny black colonies surrounded by black halos due to esculin hydrolysis within an incubation period of 24 h, wherein other bacterial species like members of enterococci form weakly brownish black coloured colonies in an incubation period of more than 40 h] were picked up and inoculated into single strength BHI broth. Inoculated tubes were incubated for 24 h at 37°C. From this BHI culture broth, one

loopful was streaked onto pre-poured plates of Listeria Oxford selective agar. Streaked agar plates were incubated for 24-48 h at 37°C.

Characteristic colonies of *Listeria* spp. as described earlier were selected at random and streaked onto pre-poured plates of nutrient agar and incubated for 24 h at 37°C. Plates with growth of culture were observed under the passage of light at an angle of 45°. Cultures of *Listeria* spp. exhibited blue-grey to blue colour. Such presumptively confirmed isolates of *Listeria* were selected, numbered and maintained at 4°C on BHI agar slants with regular subculturing once in 30 d. The isolated cultures were maintained in duplicate, with one set being used for characterization.

**4.1.2.2 Characterization of native food isolates to its species level**

The presumptive native food isolates of *Listeria* and the reference cultures (**4.1.1.2**) were subjected individually to morphological and biochemical tests following the established procedures (Cappucino and Sherman, 2004).

*4.1.2.2.1 Gram's stain*

The heat fixed smear of the individual isolates of *Listeria* prepared on a clean glass slide was stained with crystal violet for 1 min, followed by washing off excess stain with water. Then Lugol's iodine solution was added and allowed to react for 1 min. After washing off iodine with water, the smear was treated with 95% ethanol for 30 seconds, so as to remove the excess crystal violet. Finally, the smear was counter stained with safranin, allowing to react for 30 seconds, washed with water, dried and examined under oil immersion of a compound microscope. Gram positive cells appear as violet coloured, while Gram negative cells as pink coloured. Besides, the cell morphology was also recorded.

*4.1.2.2.2 Catalase production*

Test cultures were grown freshly on BHI agar slants, onto which were added few drops of 3% (v/v) hydrogen peroxide. Culture tubes were observed for the formation of nascent oxygen in the form of bubbles. This indicates positive for catalase production.

*4.1.2.2.3 Oxidase production*

A speck of freshly grown culture at 37°C was smeared on the edge of a piece of filter paper, onto which was added a drop of the reagent (1% aqueous solution of N,N,-dimethyl-p-phenylene diamine). The edge of the culture smear was observed for colour change in 30 sec. Violet colouration indicates oxidase positive reaction.

*4.1.2.2.4 Test for motility; Oxidative/fermentative reaction*

Stab inoculation of the test cultures individually were performed in the sterile tubes of Hugh Leifson medium. Each culture was inoculated in duplicate. To one set of the inoculated tubes, few drops of sterile liquid paraffin were added to overlay the agar medium (anaerobic condition). The other set of tubes were kept without any addition. Both the sets of tubes were incubated at 37°C for 24-48 h. Incubated tubes were observed for the spreading of the culture growth from the line of inoculation, which indicates the motile nature of the culture. Acid production from glucose was indicated by colour change of the medium from light green to yellow. Positive tubes for acid production under aerobic condition indicate the oxidative nature, while positive tubes under anaerobic condition indicate fermentative nature.

At the same time, cultures of *Listeria* were also tested for the 'Tumbling Motility', a unique characteristic of this bacterial organism. Test isolates were grown in BHI broth for 16-24 h at 20-25°C. A wet mount of the culture broth was prepared

and observed under a Compound Light Microscope for a gentle rotating of the cells in a tumbling motion.

*4.1.2.2.5 Methyl red and Voges Proskauer (MR-VP) reaction*

These two tests were performed using MR-VP broth. Test cultures individually were inoculated into the broth medium and incubated for 24 h at 37°C. The culture broth was divided into two parts, one part was used for MR reaction and the other for VP reaction.

Methyl red test:

Methyl red indicator was prepared by dissolving 0.1 g methyl red in 300 ml of 95% ethanol and later made upto 500 ml with distilled water. Add 5-6 drops of MR reagent to one part of the culture broth. Development of pink colour indicates positive reaction.

Voges Proskauer test:

VP test reagent consists of 2 solutions

| **Solution A** | | **Solution B** | |
|---|---|---|---|
| $\alpha$-napthol | 5 g | Potassium hydroxide | 40 g |
| Absolute alcohol | 100 ml | Creatine | 0.5 g |
| | | Distilled water | 100 ml |

To the second part of culture broth, 0.6 ml of solution A and 0.2 ml of solution B were added, mixed well and tubes kept unplugged so as to allow for the incorporation of air. Formation of eosin pink colour indicated positive reaction.

*4.1.2.2.6 Urease production*

This test was carried out using Kohn two tube medium No. 1. Individual test cultures were inoculated into the prepared slants by making a stab in the butt and streaking on the slant. Inoculated tubes were incubated for 24-48 h at 37°C. Positive urease reaction (i.e. alkaline) was indicated by a deep cerise colour of the whole medium.

*4.1.2.2.7 Indole production*

Individual test cultures were inoculated into prepared tryptone broth tubes and incubated for 24 h at 37°C. To each of the incubated tubes was added 0.2-0.3 ml of Kovac's reagent. The formation of a dark red colour in the surface layer of the culture broth indicates a positive test for indole.

Kovac's reagent:

| | |
|---|---|
| p-Dimethyl amino benzaldehyde | 5 g |
| Amyl alcohol | 75 ml |
| Conc. Hydrochloric acid | 25 ml |

Dissolve p-Dimethyl amino benzaldehyde in the amyl alcohol and then slowly add HCl.

*4.1.2.2.8 Nitrate reduction*

Individual test cultures were inoculated into the prepared nitrate broth and incubated for 24 h at 37°C. The culture broth was tested for nitrate reduction using the following reagent which consisted of two solutions:

| **Solution 1** | | **Solution 2** | |
|---|---|---|---|
| Sulphanilic acid | 8 g | $\alpha$-napthol | 5 g |
| 5N Acetic acid | 1000 ml | 5N Acetic acid | 1000 ml |

To 5 ml of the 24 h-old culture broth was added 2 drops each of solution (1) and (2). Development of orange/brick red colour was indicative of nitrate reduction to nitrite.

*4.1.2.2.9 Acid production from sugars*

To a set of 3 tubes of 3 ml each of sugar fermentation basal medium was added 0.3 ml each of the 3 individual 10% membrane filtered sugar solutions namely mannitol, rhamnose and xylose. These tubes were then inoculated with the individual test cultures, mixed well and incubated at 37°C for a period of 5 d. Incubated tubes were observed at 24 h intervals for acid production by the colour change in the medium i.e. from purple to yellow.

*4.1.2.2.10 Assay for phospholipase activity*

In general, pathogenic cultures of *L. monocytogenes* are known to be positive for the presence of phosphatidylinositol specific phospholipase C (PL-PLC) activity (also known as lecithinase activity). PL-PLC is an important virulence factor present in *L. monocytogenes* and is usually used as a marker to distinguish pathogenic from non-pathogenic cultures. Presumptive isolates of *Listeria* were spot inoculated on pre-poured plates of BHI-egg yolk agar and incubated for 24-48 h at 37°C. Incubated plates were observed for the formation of white halo surrounding the spot inoculations in the agar plates.

*4.1.2.2.11 Haemolysis in blood agar*

Individual test cultures were spot inoculated at appropriate places in pre-poured plates of blood agar and incubated for 24 h at 37°C. Incubated plates were observed for type and degree of haemolysis under bright light. The formation of clear zones either with green colour or complete colourless around the spot of inoculation indicates α- and β-haemolysis, respectively.

*4.1.2.2.12* Christic-Atkins-Munch-Peterson (*CAMP) test*

Test cultures of *Listeria* spp. and the standard strains were streaked at right angle to the haemolytic strain of *Staphylococcus aureus* on pre-poured blood agar plates. The streaked plates were incubated for 24-48 h at 37°C. Plates were observed for the haemolysis produced by the individual cultures and at the intersection between *S. aureus* and *Listeria* spp.

**4.1.3 RESULTS AND DISCUSSION**

**4.1.3.1 Prevalence of *Listeria* spp. in a diverse range of foods**

The experimental study relating to prevalence of *L. monocytogenes* and other *Listeria* spp. in milk and milk based foods, vegetables, cereal/legume-based foods,

meat and meat based foods showed a lower proportion of their occurrence in these foods samples. The protocols followed for isolation and characterization of isolates by the conventional morphological, cultural and biochemical characteristics revealed that only 13 out of 52 samples of raw cow and buffalo milk were positive for presumptive *Listeria* spp. wherein isolates obtained in these samples were positive for esculin hydrolysis and exhibited black coloured colonies as well as tumbling motility when grown at $25^0$C. On completing the requisite characterization tests, all presumptive samples had the presence of confirmed isolates of *Listeria* spp.

The non-selective cold enrichment used in the present study resulted in the isolation of 31 isolates of *Listeria* spp. from 52 samples of raw milk (26%). Isolates of *L. grayi* was the predominating *Listeria* spp. among the isolates showing the prevalence to an extent of 83.9%. In milk samples, there was no prevalence of *L. monocytogenes* and other *Listeria* spp. Although this method was more or less similar to USFDA method, it includes certain additional steps of enrichment and plating on *Listeria* oxford agar base. This was undertaken, so as to recover the isolates of *Listeria* spp., which may be present in low numbers. The isolates obtained from samples of raw milk were characterized by morphological and biochemical tests and presented in **Table 1**.

Prevalence of *Listeria* spp. in milk-based products was 13.3%. Two out of 15 samples of different milk-based foods were positive for presumptive *Listeria* spp. The enrichment method used in the present study resulted in obtaining 8 isolates from of milk powder and ice cream. The prevalence of *L. monocytogenes* and *L. grayi* was 37.5%, each and *L. ivanovii* was 25%. The isolates were characterized by morphological and biochemical tests and shown in **Table 2.**

Raw meat from poultry, fish and sheep showed 20% of the samples harboured *Listeria* spp*.* Cultures of *L. ivanovii* predominated among the 8 isolates obtained from 40 raw meat samples, while meat-based products did not result in the isolation of any *Listeria* spp. Characteristics of the presumptive *Listeria* isolates were obtained from raw meat samples are presented in **Table 3.**

Raw vegetable samples showed the prevalence of *Listeria* spp. to an extent of 10.9% (6 out of 55 samples). Among the *Listeria* isolates, *L*. *grayi* predominated to an extent of 22%, followed by *L*. *ivanovii* (16.6%). Isolates of *L*. *monocytogenes* was found to have a very low incidence of 5.5%. Few unconventional *Listeria* spp. obtained by the cold enrichment method were positive for esculin hydrolysis and tumbling motility (55% of isolates). The characteristics of these isolates are shown in **Table 4.**

In order to determine the β-haemolytic character of individual *Listeria* isolates, a heavy growth was spot inoculated on blood agar plates. It was observed, that 4 isolates from milk-based food samples, 7 isolates from raw meat and 4 isolates from vegetables showed β-haemolysis. Further confirmation of these isolates was done by CAMP test with *S. aureus* on blood agar plate (**Figure 1**). Three isolates showed synergistic enhanced haemolysis near to the streak of *S. aureus* indicating the isolates were those of *L. monocytogenes*. In order to determine the phospholipase character of *Listeria* isolates, a heavy growth was spot inoculated on BHI-egg yolk agar plates. Five isolates from milk-based food samples and 2 isolates from vegetables showed phospholipase activity. The distribution pattern of characterized isolates of *L. monocytogenes* and other *Listeria* spp. among the analyzed food samples are presented in **Table 5**.

**Table 1** Characterization of native isolates of *Listeria* species from raw milk

| Native Isolate | Phospholipase production | CAMP test | Sugar utilisation | | | Identified culture |
|---|---|---|---|---|---|---|
| | | | Mannitol | Rhamnose | Xylose | |
| M 01 | - | - | + | - | - | *L. grayi* CFR 1310 |
| M 02 | - | - | + | - | - | *L. grayi* CFR 1311 |
| M 03 | - | - | + | - | - | *L. grayi* CFR 1312 |
| M 04 | - | - | + | - | - | *L. grayi* CFR 1313 |
| M 05 | - | - | + | - | - | *L. grayi* CFR 1314 |
| M 06 | - | - | + | - | - | *L. grayi* CFR 1315 |
| M 07 | - | - | + | + | - | *L. grayi* CFR 1316 |
| M 08 | - | - | + | + | - | *L. grayi* CFR 1317 |
| M 09 | - | - | + | ± | - | *L. grayi* CFR 1318 |
| M 10 | - | - | + | ± | - | *L. grayi* CFR 1319 |
| M 11 | - | - | + | ± | - | *L. grayi* CFR 1320 |
| M 12 | - | - | + | + | - | *L. grayi* CFR 1321 |
| M 13 | - | - | + | ± | - | *L. grayi* CFR 1322 |
| M 14 | - | - | + | ± | - | *L. grayi* CFR 1323 |
| M 15 | - | - | + | - | - | *L. grayi* CFR 1324 |
| M 16 | - | - | + | - | - | *L. grayi* CFR 1325 |
| M 17 | - | - | + | - | - | *L. grayi* CFR 1326 |
| M 18 | - | - | + | - | - | *L. grayi* CFR 1327 |
| M 19 | - | - | + | - | - | *L. grayi* CFR 1328 |
| M 20 | - | - | + | - | - | *L. grayi* CFR 1329 |
| M 21 | - | - | + | - | - | *L. grayi* CFR 1330 |
| M 22 | - | - | + | - | - | *L. grayi* CFR 1331 |
| M 23 | - | - | + | - | - | *L. grayi* CFR 1332 |
| M 24 | - | - | + | - | - | *L. grayi* CFR 1333 |

All the isolates were positive for esculin hydrolysis, Gram's reaction, catalase production; exhibited tumbling motility, Methyl Red and Voges Proskauer reactions; and negative for oxidase, indole and urease production as well as nitrate reduction

**Table 2** Characterization of native isolates of *Listeria* species from milk based foods

| Native Isolate | Phospholipase production | CAMP test | Sugar utilisation | | | Identified culture |
|---|---|---|---|---|---|---|
| | | | Mannitol | Rhamnose | Xylose | |
| M 25 | + | + | + | + | - | *L. monocytogenes* CFR 1334 |
| M 26 | + | + | + | + | - | *L. monocytogenes* CFR 1335 |
| M 27 | + | - | + | + | + | *L. ivanovii* CFR 1336 |
| M 28 | + | + | + | + | + | *L. ivanovii* CFR 1337 |
| M 29 | + | + | + | + | - | *L. monocytogenes* CFR 1338 |
| M 30 | - | - | + | - | - | *L. grayi* CFR 1339 |
| M 31 | - | - | + | - | - | *L. grayi* CFR 1340 |
| M 32 | - | - | + | - | - | *L. grayi* CFR 1341 |

All the isolates were positive for esculin hydrolysis, Gram's reaction, catalase production; exhibited tumbling motility, Methyl Red and Voges Proskauer reactions; and negative for oxidase, indole and urease production as well as nitrate reduction

**Table 3** Characterization of native isolates of *Listeria* species from raw meat samples

| Native Isolate | Phospholipase production | CAMP test | Sugar utilisation | | | Identified culture |
|---|---|---|---|---|---|---|
| | | | Mannitol | Rhamnose | Xylose | |
| M 33 | - | + | + | + | + | *L. ivanovii* CFR 1342 |
| M 34 | - | + | + | + | + | *L. ivanovii* CFR 1343 |
| M 35 | - | + | + | + | + | *L. ivanovii* CFR 1344 |
| M 36 | - | + | + | + | + | *L. ivanovii* CFR 1345 |
| M 37 | - | + | + | + | + | *L. ivanovii* CFR 1346 |
| M 38 | - | + | + | + | + | *L. ivanovii* CFR 1347 |
| M 39 | - | + | + | + | + | *L. ivanovii* CFR 1348 |

All the isolates were positive for esculin hydrolysis, Gram's reaction, catalase production; exhibited tumbling motility, Methyl Red and Voges Proskauer reactions; and negative for oxidase, indole and urease production as well as nitrate reduction

**Table 4** Characterization of native isolates of *Listeria* species from raw vegetables

| Native Isolate | Phospholipase production | CAMP test | Sugar utilisation | | | Identified culture |
|---|---|---|---|---|---|---|
| | | | Mannitol | Rhamnose | Xylose | |
| M 01 | - | - | + | ± | - | *L. grayi* CFR 1349 |
| M 02 | - | - | + | + | - | *L. grayi* CFR 1350 |
| M 03 | - | - | + | + | + | *L. grayi* CFR 1351 |
| M 04 | - | - | + | + | + | *L. grayi* CFR 1352 |
| M 05 | ± | + | + | + | + | *L. ivanovii* CFR 1353 |
| M 06 | - | - | + | + | + | *Listeria* sp. CFR 1354 |
| M 07 | ± | + | + | + | - | *L. monocytogenes* CFR 1355 |
| M 08 | - | - | + | + | + | *Listeria* sp. CFR 1356 |
| M 09 | - | - | + | - | - | *Listeria* sp. CFR 1357 |
| M 10 | - | - | + | - | + | *Listeria* sp. CFR 1358 |
| M 11 | - | - | + | - | - | *Listeria* sp. CFR 1359 |
| M 12 | - | - | + | + | + | *Listeria* sp. CFR 1360 |
| M 13 | - | - | + | + | + | *Listeria* sp. CFR 1361 |
| M 14 | - | - | + | + | + | *Listeria* sp. CFR 1362 |
| M 15 | - | - | + | + | + | *Listeria* sp. CFR 1363 |
| M 16 | + | + | + | + | + | *L. ivanovii* CFR 1364 |
| M 17 | - | - | + | + | + | *Listeria* sp. CFR 1365 |
| M 18 | + | + | + | + | + | *L. ivanovii* CFR 1366 |

All the isolates were positive for esculin hydrolysis, Gram's reaction, catalase production; exhibited tumbling motility, Methyl Red and Voges Proskauer reactions; and negative for oxidase, indole and urease production as well as nitrate reduction

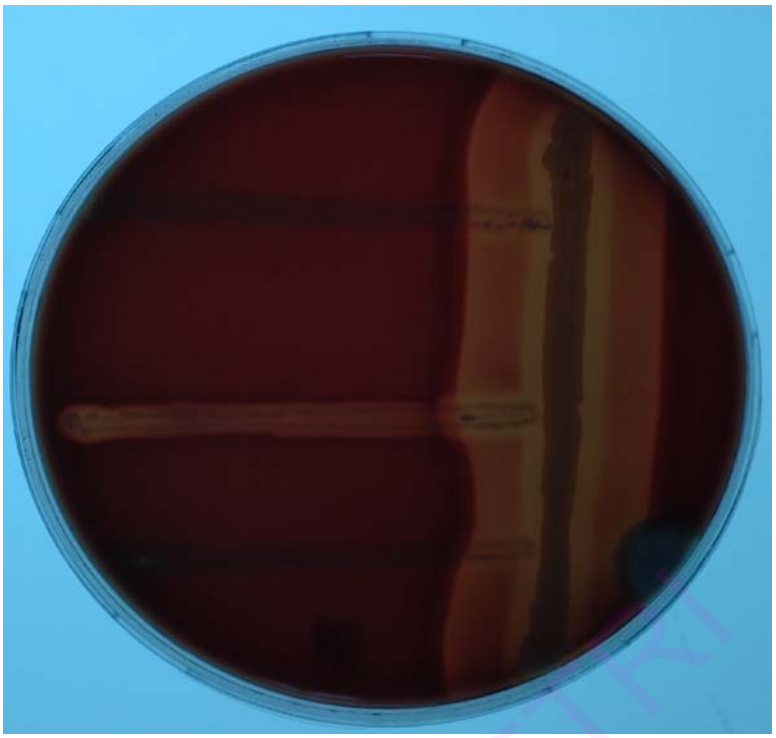


**Figure 1** Native food isolate of *L*. *monocytogenes* CFR 1338 showing positive reaction in CAMP test (synergistic haemolysin activity with *S*. *aureus*)

**Table 5** Distribution of characterized isolates of *Listeria spp.* among the analysed food samples

| Samples analyzed Type / Nos. | Samples positive for *Listeria* sp. (n) / (%) | Identified isolates of *Listeria* sp. Type / Nos. |
|---|---|---|
| Raw whole milk / 50 | 13 / 26.0 | *L. grayi* / **24** |
| Milk-based foods / 15 | 02 / 13.3 | *L. monocytogenes* / **03** |
| | | *L. ivanovii* / **02** |
| | | *L. grayi* / **03** |
| Raw meat / 40 | 08 / 20.0 | *L. ivanovii* / **07** |
| Meat-based products / 10 | Nil | Nil |
| Cereal/legume-based products / 08 | Nil | Nil |
| Raw vegetables / 55 | 06 / 10.9 | *L. grayi* / **04** |
| | | *L. monocytogenes* / **01** |
| | | *L. ivanovii* / **03** |
| | | *Listeria* sp. / **10** |

Earlier, Kamat and Nair (1994) used this non-selective cold enrichment and were able to enumerate a large population of *Listeria* spp. from samples of fish and meat. They isolated *L. grayi* and *L. seeligeri* in fish and *L. ivanovii, L. seeligeri and L. welshimeri* from meat samples. A similar cold enrichment technique was used by Hayes et al. (1986), wherein *L. monocytogenes* was recovered from 15 (12%) of 121 milk samples and 2 (14%) of milk stocks.

The non-selective cold enrichment was the efficient method for best recovery of *Listeria* from milk samples. Direct plating, selective enrichment and cold enrichment in a non-selective medium, followed by secondary selective enrichment procedures were compared by Hao et al. (1987) and they report cold enrichment in BHI broth, followed by secondary enrichment and plating on selective media resulted in the detection of highest population of *Listeria* in refrigerated cabbage. The samples of cheese, butter, pasteurized and raw milk sold in Mumbai market were analysed for *Listeria* spp by both cold enrichment and the USFDA method by Pednekar et al. (1997). They have found cold enrichment technique as superior to recover *Listeria* spp. from raw milk and ice cream. The *Listeria* spp. were not recovered from the samples of cheese and butter by both the methods. Jayashekaran et al. (1995) used the protocol including pre-enrichment, followed by two selective enrichments and plating on three selective agars and recovered *L. monocytogenes* from different samples of fish (29.2%) and *L. innocua* (12.5%).

Slade and Collins-Thompson (1988) compared both direct enrichment at $30^0$C, as well as two-stage enrichment and reports both the methods were equally effective in isolating *Listeria* spp. Bhilegaonkar et al. (1997) used direct enrichment for isolation of *Listeria* from milk using University of Vermont Broth I (UVM-I) at $30^0$C for 48 h, followed by transferring to UVM Broth II and subsequent plating on selective agar plates (Dominguez-Rodriguez isolation agar). They were able to recover 7.44%

of *Listeria* spp. from 121 milk samples, whereas in the present study there was only 26% recovery of *Listeria* spp*.* from 65 samples of milk and milk products.

In a large survey on *Listeria* spp*.* in bovine raw milk undertaken by Kalorey et al. (2008) in Central India, *Listeria* spp*.* were isolated using direct enrichment and plating on selective agar as per the USDA method. Isolates of *Listeria* spp*.* were obtained from 139 (6.75%) milk samples collected from 2060 dairy cows. From these reports, it may be concluded that the prevalence of *Listeria* spp. and *L. monocytogenes* in raw cow milk was 9.2% and 3.0%, respectively, in our study, which was lower than that reported by Bhilegaonkar, wherein it was 13.2%. Similarly, Barbudde et al. (2002) reported 26.1% prevalence of *Listeria* spp. in Northern India.

Warke et al. (2000) isolated *Listeria* spp. from ice cream samples by non-selective cold enrichment in which 25 ml of ice cream (molten) was inoculated in 25 ml of double strength BHI broth, followed by selective enrichment. The packed ice cream samples (53%) and open ice cream samples (100%) exhibited *Listeria* contamination and *L. monocytogenes* was recovered from one of the open ice cream samples. In Turkey, *Listeria* spp*.* were isolated from 41.4% raw milk and 37.9% of ice cream samples (Akman et al. 2004). In our study, *L. grayi* was isolated from one ice cream sample.

The incidence of *Listeria* in *Peda* samples prepared from *Khoa* was studied and it was found 33% of *Peda* samples form 'B' Grade shops showed the presence of *Listeria, Yersinia* and *coliforms.* (Bandekar et al. 2000). Incidence and seasonal variation of *Listeria* spp. in bulk tank goat's milk was analyzed for over one year period. From the 39 goat farms, 7.8 % samples yielded *Listeria spp.* with *L. monocytogenes* occurring to an extent of 3.8% (Abou-Eleinin et al. 2000). The prevalence of *L. monocytogenes* and other *Listeria spp.* was studied in 329

European read smear cheese and the result showed that the cheeses were contaminated with *Listeria spp.* (15.8%) and *L. monocytogenes* 6.4% (Rudolf and Scherer, 2001).

The prevalence of *L. monocytogenes* from milk and milk farm products such as butter, yoghurts, cheeses, ice cream and fresh cheeses showed that *Listeria* was prevalent in all types of products in low numbers in Belgium (Rue et al. 2004). A comprehensive survey was done to know the presence of *L. monocytogenes* in pasteurized milk produced in the USA. Nearly 5519 samples which included whole milk, skim milk, chocolate milk were analyzed for the prevalence of *L. monocytogenes*. The frequency of isolation from these products was very less and the results confirmed the low frequency of contamination of pasteurised milk by *L. monocytogenes* was very low (Frye and Donelly, 2005). Prevalence of *L. monocytogenes* in bulk tank milk in United States dairies obtained from farms in 21 states revealed that only 6.5% bulk tank milk samples among 861 bulk tank milk samples contained *L. monocytogenes* (Van Kesse et al. 2004). In our study, milk-based products like milk powder revealed the prevalence of L. monocytogenes to an extent of 37.5%.

Pingulikar et al. (2001) have surveyed 116 samples from 11 different fresh vegetables from local markets, which were consumed in raw form, as well as 12 samples of ready-to-eat green salads from restaurants of Mumbai, India for the incidence of *Listeria* spp. and *Yersinia*. Nearly, 100% of the local vegetables and 73% vegetable salads exhibited the incidence of *Listeria* spp. Isolates of *L. monocytogenes* was observed in 11.3% of tomatoes, 50% of coriander leaves, 50% of spinach and 25% from cabbage samples. Dhanashree et al. (2003) have investigated 633 clinical and 320 food samples from Mangalore, India and isolated *L. monocytogenes* from two clinical and two food samples. The incidence of *Listeria*

spp. was 17.5 % in food samples, whereas *L. innocua* predominated in leafy vegetables to an extent of 30%. In our studies, vegetables showed 10.9% of incidence of *Listeria* spp*.* An isolate of *L. monocytogenes* was present in a sample of beet root and that of *L.ivanovii* in cabbage.

The prevalence of *Listeria* spp. in tropical fish and fish products was carried out by Fuchs et al. (1989). The study showed only *L. innocua* from fish and sea foods upto 30%. Drake et al. (2005) identified *Listeria* spp. and *L. monocytogenes* from aquacultured cat cannel fish upto 58% and 2%, respectively. In our studies, none of the fish samples showed the presence of *Listeria* spp*.*

Occurrence of *L. monocytogenes* in meat was studied by Barbuddhe et al. (2002) by using UVM medium and plating on Dominguez-Rodriguez isolation agar. Of the 167 meat samples, 2.4 and 10.17% were positive for *L. monocytogenes* and *Listeria* spp., respectively. Antunes et al. (2002) has investigated 63 samples of poultry carcasses in Portugal. The poultry samples were contaminated with 26% of *Listeria* spp. including *L. monocytogenes, L. innocua, L. seelgeri* and *L. welshimeri*. Occurrence and distribution of *Listeria* spp. was studied in meat processing plants by Samelis et al. (1998). *Listeria monocytogenes* and other *Listeria* spp. were found to an extent of 51% and 49%, respectively, in frozen raw meat. In our study, 20% of raw meat showed the presence of *Listeria* spp., whereas *L. ivanovii* was predominating almost to an extent 100%

From the above findings, it can be concluded, that listerial contamination of foods was prevalent in various proportions in different products, world-wide devoid of any barriers. The main source of *Listeria* in raw milk was attributed to fecal contamination and post-milking contamination during storage and transport (Griffiths, 1989). Hence, the prevalence of *Listeria* spp. in raw milk and milk products depends

upon a number of factors which includes the health conditions of milking animals, quality of the feed used, hygienic conditions in the milking area and of the equipments and other factors. The presence of *Listeria* spp. in meat was mainly due to the processing units, wherein pre contaminated meat have been used (Samelis et al. 1999).

Considering the level of incidences of *Listeria spp.* in food, there occur ample opportunities, that it may be transferred to humans. In this background, it becomes necessary to maintain strict hygienic conditions and practice of pasteurisation of milk in order to minimise human listeriosis. Our study shows very low incidence rate of *L. monocytogenes* in food, supports the findings that listeriosis is an uncommon infection in present area.

In the present global situation, the findings of a few of these studies cannot be taken as a whole representative of the Indian scenario, because of the size of the samples analysed in relation to the geographical nature of our country. The prevailing situation makes it more meaningful to have more number of studies relating to the incidence and characterization of *Listeria* spp. in the Indian scenario.

## 4.2 IMMUNOLOGICAL METHODS FOR THE DETECTION OF *LISTERIA MONOCYTOGENES*

The occurrence of *L*. *monocytogenes* in foods has been a cause of health concern and detection of this pathogenic organism from foods is still a challenging task for the food microbiologists. The conventional culture dependent enrichment based plating methods are time consuming and labour extensive. Regulations limiting contamination of foods to a zero tolerance policy have been the driving force behind development of faster, reliable and specific methods. In this direction, attempts have been in progress to develop and/or standardize immunoassays. Immunoassays are based on natural affinity of antibodies towards antigen. The antigen can be a protein or carbohydrate. The accurate detection of the organism is acquired by selecting the specific antigen, development of antibody and development of assay. Whole cells, extracellular proteins and intracellular proteins are routinely used as antigens. Listerial proteins like flagellar proteins, listeriolysin O, phospholipase C, metallo-proteins, actin polymerizing proteins and several functionally unknown proteins are being used over the years to detect *L. monocytogenes*. Immunoassays based on antibodies specific to Listeria spp. have been in use to assess the safety of foods from *L*. *monocytogenes* and the results have been highly varied.

In the above background, the present experimental approach attempts to partially purify and characterize listeriolysin (LLO), raise antibodies against LLO in rabbits and poultry and use the prepared antibodies to detect *Listeria* spp. by ELISA and Dot Blot techniques in pure culture system and food matrix.

### 4.2.1 MATERIALS

All the glasswares used in the present investigation were either sterilized by autoclaving at 121°C for 20 min or by dry sterilization at 180°C for 4 h in a hot-air oven. Gamma-irradiated sterile disposable petri plates, and dehydrated culture media procured from HiMedia laboratories, Mumbai, India. The media were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 min. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

The specific requisites in respect of specific fine biochemicals and reagents for immunoassays, protein isolation, purification and molecular weight determinations such as Biogel P-100, Sephadex G-75, Acrylamide, bis-acrylamide, sodium lauryl sulphate, TRIS buffer, TEMED, Ammonium per sulphate, bromophenol blue, ß-mercaptoethanol, Comassie Blue G 250, related buffers, reagents and solvents used in this experimental study were obtained from Sigma-Aldrich, Bangalore, India; Bangalore Genei, Bangalore, India; Sisco Research Laboratories, Mumbai, India; and Qualigens, Mumbai, India.

#### 4.2.1.1 Bacterial cultures and inoculum preparation

This included the Reference Cultures as described under **4.1.1.2**. Besides, a few selected cultures of foodborne pathogenic bacterial species and a native food isolate of *L*. *monocytogenes* CFR 1338 were also included (**Table 6**). The cultures were maintained at 4°C on brain heart infusion (BHI) agar slants and subcultured at 15 d intervals. The cultures were propagated twice in BHI broth at 37°C prior to use in experiment.

**Table 6** Test cultures used in this experimental study

| Culture | Source |
|---|---|
| *Listeria monocytogenes* Scott A, *L. monocytogenes* V 7 and *L*. *innocua* Prd. 01 | Dr. Arun K. Bhunia, Purdue University, United States of America |
| *Listeria ivanovii* Mng 01, *L*. *seeligeri* Mng 02, *L*. *grayi* subsp. *grayi* Mng 03 and *L*. *grayi* subsp. *murrayi* Mng 04 | Dr. I. Karunasagar, College of Fisheries, KVAFS University, Mangalore, India |
| *Escherichia coli* MTCC 118 and *Yersinia enterocolitica* MTCC 859 | Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India |
| *Bacillus cereus* F 4810 | Dr. J.H. Kramer, Public Health Laboratory, Colindale, United Kingdom |
| *Staphylococcus aureus* FRI 722 | Dr. S. Notermans, Public Health Laboratory, The Netherlands |
| *L*. *monocytogenes* CFR 1338 | Native isolate from milk powder (from this study - **Table 2 of 4.1**) |

Prior to use in experiments, the individual cultures of *L*. *monocytogenes* Scott A, *L*. *monocytogenes* CFR 1338 and *L*. *innocua* Prd. 01 were successively propagated twice in BHI broth for 24 h at 37°C in an orbital shaker incubator (Alpha Scientific Co., Bangalore, India) at 140 rpm. Cell suspensions of these cultures were individually prepared from 20 h old BHI culture broth (grown under conditions previously described) by centrifugation at 8000 rpm for 20 min at 4°C (Superspin R-V/$F_M$, Plasto Crafts, Mumbai, India). The harvested cells were resuspended in sterile 10 ml aliquots of 0.85% saline and stored in sterile screw-capped tubes at 4°C until further use.

#### 4.2.1.2 Diluents

The diluents used were as follows:

i. Requisite quantities of normal saline of 0.85% were dispensed in suitable glass containers and autoclaved.

ii. Potassium phosphate buffer (PPB) of 0.01 M

The stock solution of 0.1 M was prepared as follows:

| Vol. of 1 M $K_2HPO_4$ | Vol. of 1 M $KH_2PO_4$ | Resulting pH |
|---|---|---|
| 4.25 | 45.75 | 5.8 |
| 13.9 | 36.1 | 6.4 |
| 30.75 | 19.25 | 7.0 |
| 43.3 | 6.7 | 7.6 |
| 47.0 | 3.0 | 8.0 |

The stock solution was prepared by dissolving the above ingredients in Milli-Q water and the volume was made up to 1000 ml in a volumetric flask and stored at 4°C till further use. For use in the experimental trials, the stock solution was diluted in the ratio of 1:9 with Milli-Q water, pH adjusted with either 0.1 N HCl and/or NaOH, if necessary. The prepared PPB was dispensed in requisite quantities into appropriate glasswares and autoclaved at 121°C for 15 min.

**4.2.1.3 Bacteriological media**

*4.2.1.3.1 Brain heart infusion (BHI) broth and agar*

This was prepared as described under **4.1.1.5.2** (**A**) and used in this study.

*4.2.1.3.2 Nutrient broth and agar*

This was prepared as described under **4.1.1.5.11** and used in this study.

*4.2.1.3.3 Plate count agar*

This was prepared as described under **4.1.1.5.12** and used in this study.

*4.2.1.3.4 Listeria Oxford Agar*

This was prepared as described under **4.1.1.5.6**.

#### 4.2.1.4 Skim milk

This was prepared by reconstituting requisite quantities of spray dried skim milk powder (Sagar Brand, Gujarat Cooperative Milk Marketing Federation Ltd., Anand, India). The reconstituted milk was filtered through moistened cotton pad, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10 ml amounts in test tubes (18 x 150 mm) and autoclaved at 121°C for 15 min.

#### 4.2.1.5 Bradford reagent

This was prepared as follows:

| | |
|---|---|
| Comassie brilliant blue G 250 | 100 mg |
| Ethanol | 50 ml |
| Phosphoric acid (85% w/v) | 100 ml |

The above were mixed well and volume made up to 1000 ml in a volumetric flask, filtered and stored in brown bottle under dark conditions for use in the experimental trials.

### 4.2.2 METHODOLOGY

***All experimental trials were carried out independently, in triplicates / duplicates, as the case may be and mean values with requisite statistical analysis were applied in presenting the results.***

#### 4.2.2.1 Determination of protein content

In the present experimental trials, protein was quantified in samples by Bradford method (reference). To suitably diluted samples (1.5 ml), equal volume of Bradford reagent was added and mixed well. The mixed solution was incubated at ambient temperature for 30 min with intermittent shaking, following which absorbance was read at 595 nm in a UV-VIS Spectrophotometer (UV-160A, Shimadzu Corporation, Kyoto,

Japan). A standard graph was prepared with different requisite concentrations of bovine serum albumin.

#### 4.2.2.2 Preparation of red blood cells (RBC)

Requisite quantities of blood from rabbits and human volunteers were collected into suitable pre-sterile glass containers with 10% of 0.2 M EDTA. The collected blood samples were centrifuged at 5000 rpm for 10 min at 4°C in a refrigerated centrifuge (Rota 4R-V/$F_M$, Plasto Crafts, Mumbai, India). The resulting RBCs were washed 2-3 times with normal saline, till the upper supernatant became clear. The purity of RBC was checked by microscopic observation and stored at 4°C till further use within a period of 6days from the time of preparation.

#### 4.2.2.3 Determination of haemolytic activity

Haemolytic activity was assayed following the method described by Portnoy et al.. (1988). One volume of RBC was diluted with 100 volumes of normal saline. Aliquots of 750 ul of test sample (mostly protein) were incubated with 750 ul of RBC for 30 min at 37°C. The mixture was centrifuged at 5000 rpm for 5 min at 4°C in a refrigerated centrifuge (Rota 4R-V/$F_M$) and read for absorbance at 450 nm in a UV-VIS Spectrophotometer (UV-160A, Shimadzu). Haemolytic units were expressed as the reciprocal of the highest dilution showing complete haemolysis. Complete haemolysis was observed by adding 10% Triton X-100 (1/100 volume) to the sample.

#### 4.2.2.4 Preliminary assessment of listeriolysin O (LLO) in cultures of *Listeria* spp.

As a preliminary assessment of the potency of cultures of Listeria spp. to elaborate virulence / pathogenic trait like listeriolysin O (LLO) as determined by haemolytic activity,

cultures of *L*. *monocytogenes* Scott A, *L*. *monocytogenes* CFR 1338 and *L*. *innocua* Prd. 01 were grown for 24 h in 100 ml aliquots of BHI broth at 37°C in a shaker incubator maintained at 120 rpm (Alpha Scientific Co., Bangalore, India). Incubated cultures were centrifuged at 8000 rpm for 20 min at 4°C (Refrigerated Centrifuge). An aliquot of the resulting cell free supernatant was used to carry out the haemolytic assay. The other aliquot of supernatant was subjected to precipitation of protein by adding equal volume of chilled acetone and allowed to stand for 18 h. Subsequently, the precipitated protein was separated by centrifugation at 8000 rpm for 20 min at 4°C. The resulting precipitate was dissolved in sterile Milli-Q water and stored in sterile screw-capped test tubes at 4°C for further use in experimental trials.

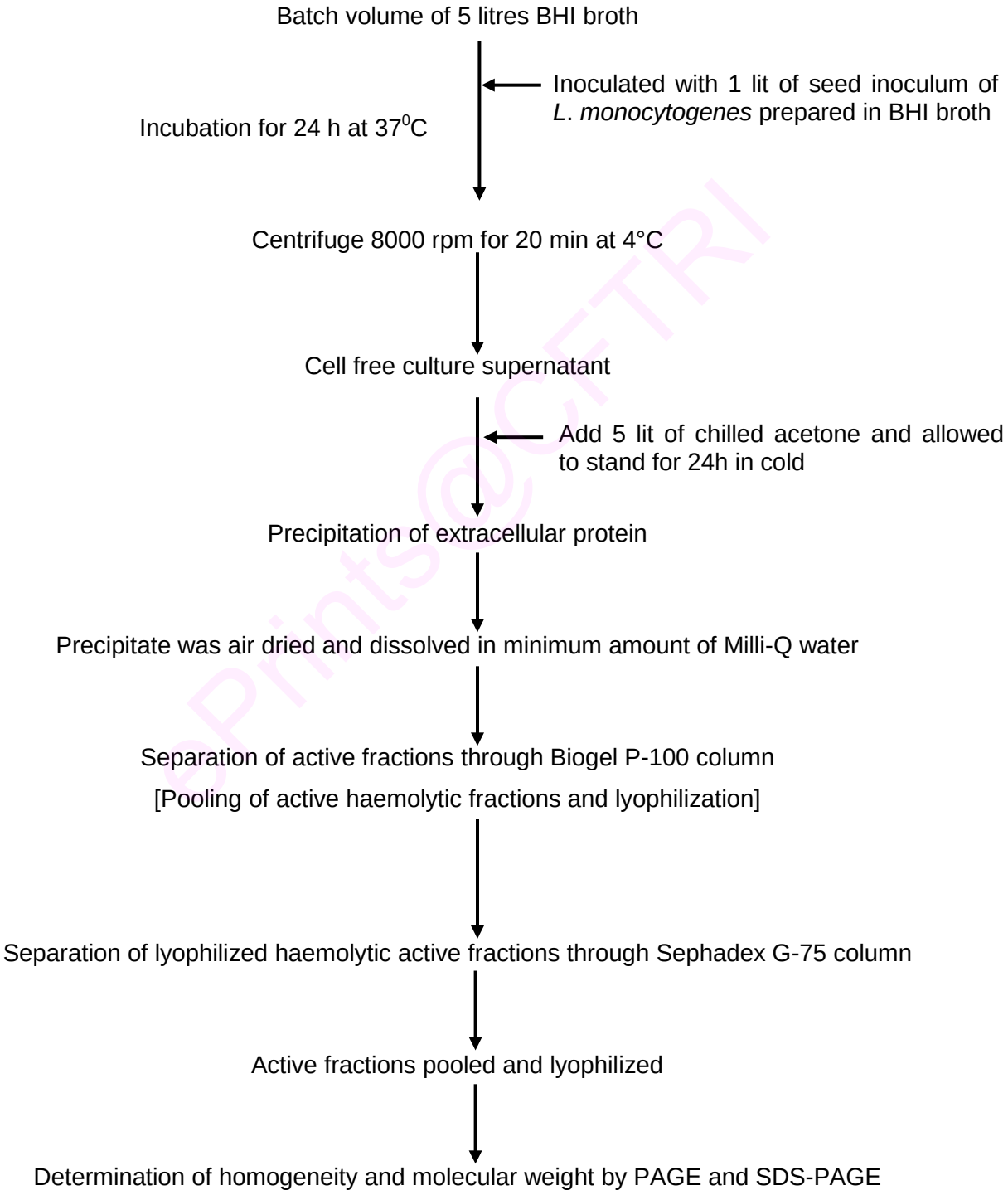
In another set of experimental trials, aliquots of BHI culture broth grown for a period of 72 h at 37°C under shaking conditions of 120 rpm were drawn at different intervals of time period like 6, 12, 18, 24, 36, 48, 60 and 72 h. The samples drawn at time bound intervals were centrifuged at 8000 rpm for 20 min at 4°C and the resulting cell-free clear supernatants were subjected to haemolytic assay by recording changes in absorbance read at 450 nm in a UV-VIS Spectrophotometer.



**4.2.2.5 Partial purification and characterization of LLO**

Based on the results of preliminary assessment for LLO among the 3 cultures of Listeria spp., it was planned to use the reference culture of *L*. *monocytogenes* Scott A to obtain an almost purified LLO for use to develop immunoassays to detect specifically *L*. *monocytogenes* in culture and food systems. Initially, an aliquot of 100 ml of BHI broth was inoculated from a freshly subcultured agar slant of *L*. *monocytogenes* Scott A and incubated for 24 h at 37°C under shakier conditions of 120 rpm. The resulting culture broth was centrifuged at 8000 rpm for 20 min at 8°C (Refrigerated Centrifuge) and the

resultant cell sediment was used to build the seed inoculum in 1000 ml of BHI broth for further inoculation to a batch volume of 5 litres of BHI broth. The protocol used for the growth of *L*. *monocytogenes* and thereafter the steps of isolation, partial purification and characterization of LLO are presented in the following flow sheet:



*4.2.2.5.1 Separation through Biogel P-100 column*

The acetone precipitated protein in 100 mg quantity was loaded to Biogel P-100 column (1.5 x 100 cm), which was pre-equilibrated with potassium phosphate buffer of pH 5.5. Individual fractions of 2 ml each were collected at a flow rate of 24 ml/60 min. The resultant fractions were evaluated for protein content by reading the absorbance at 280 nm in a UV-VIS Spectrophotometer (UV-160 A, Shimadzu). At the same time, haemolytic activity was determined only in those fractions, which formed peaks and shoulder of peaks. Subsequently, active fractions in respect of haemolytic activity were pooled and lyophilized in a laboratory model Lyophilizer (Heto Drywinner, Jouan Nordic, Allerod, Denmark) and stored in pre-sterilized screw-capped tubes at 4°C for further use in experimental trials.

*4.2.2.5.2 Separation through Sephadex G-75 column*

Lyophilized pooled active fractions obtained from Biogel P-100 column with a concentration of 60 mg protein content was loaded into Sephadex G-75 column (1.5 x 50 cm), which was pre-equilibrated with PPB of pH 5.5. Individual fractions of 1 ml each were collected at a flow rate of 24 ml/60 min. Considering that peak fractions were positive for haemolytic activity, the resultant fractions forming peaks and its shoulders were determined for protein content as described previously at an absorbance of 280 nm in UV-VIS Spectrophotometer. Only those fractions exhibiting haemolytic activity were pooled and lyophilized and stored in pre-sterilized screw-capped tubes at 4°C for further use in experimental trials.

*4.2.2.5.3 Homogeneity and molecular weight determination of purified active principle*

The lyophilized preparation of pooled active fractions obtained from Sephadex G-75 column was subjected to SDS-PAGE according to the method of Laemelli (1970)

15% acrylamide gel. Electrophoresis was performed in buffer under a constant current of 3.0 V/h. Appropriately run gels were stained with Comassie brilliant blue R 250 and destained to observe for the bands. Molecular weight was determined by comparing with standard molecular protein marker.

*4.2.2.5.4 Determination of optimum pH and temperature for the activity of LLO*

The lyophilized preparation of pooled active fractions obtained from Sephadex G-75 column was assayed for haemolytic activity with human RBC diluted in PPB of pH levels ranging from 5.5 to 8.0 at incubation temperature of 37°C for 30 min. The haemolytic activity was recorded as changes in absorbance read at 450 nm in a UV-VIS Spectrophotometer and the degree of haemolysis was calculated. Similarly, the lyophilized active fraction was evaluated for optimum temperature by carrying out the assay with rabbit RBCs diluted in PPB of pH 5.5 for 30 min at temperatures of 5, 10, 24, 30, 37, 45 and 50°C. The haemolytic activity was determined as described previously.

**4.2.2.6 Antibody (IgG) production for LLO in rabbits**

*4.2.2.6.1 Immunization protocol*

Female New Zealand white rabbit weighing 1-1.5 kg was immunized with 100 ug of purified active fraction of LLO in Milli-Q water emulsified with 0.5 ml of Freund's complete adjuvant (FCA). Immunization of rabbit was through intramuscular injections at multiple sites. Subsequent booster injections of LLO (100 ug, 200 ug, 300 ug and 400 ug protein levels) in Freund's incomplete adjuvant (FICA) were given at 30 d intervals. The trial bleeding was done after 7 d of first booster injection. The successive bleeding was carried out every 15 d of each booster injections.

*4.2.2.6.2 Isolation and purification of IgG*

Purification of IgG was carried out as described by Tissen (1985). Serum was separated from blood by low speed centrifugation and immunoglobulin (IgG) fraction was isolated by precipitation in saturated ammonium sulphate (66%) and dialyzed extensively against water and stored in sterile screw-capped tubes at –20°C till further use. Prior to storage at -20°C, the protein content was determined by Bradford method as described earlier.

**4.2.2.7 Antibody (IgY) production for LLO in poultry**

*4.2.2.7.1 Immunization protocol*

Female Hens 27 weeks old, weighing 1-1.5 kg were used in immunization experiment. Hens were immunized with 100 ug of purified active fraction of LLO in Milli-Q water emulsified with Freund's complete adjuvant. Immunization of hens was through intramuscular route, wherein injections were given at multiple sites into the pectoral muscles. Subsequent booster injections were given at monthly intervals with increasing dosage of antigen (100 ug, 200 ug, 300 ug and 400 ug). The eggs were collected after 7 days of first booster injection.

*4.2.2.7.2 Isolation and purification of IgY*

The egg yolk antibodies were isolated and purified by the method of Clarke et al.. (1993). The egg yolk was separated from the albumin and diluted with 4 volumes of 0.1mM PBS of pH 7.2 and 1 volume of chloroform. The mixture was centrifuged at 10000 rpm for 30 min at 4°C (Refrigerated Centrifuge). The resultant supernatant was collected and IgY was precipitated twice with 14% w/v PEG 6000. The IgY was reconstituted to original volume with 0.1 mM PBS containing 0.005% sodium azide. The total protein content in the antibody was measured by Bradford method. The prepared

antibodies were stored in sterile screw-capped tubes at -20°C until further use in the experimental trials.

#### 4.2.2.8 Indirect dot binding ELISA

Purified active fraction of LLO (antigen) of appropriate concentrations was prepared. Aliquots of 2 ul of antigen were spotted equidistantly on nitrocellulose strips of 30 mm (L) x 5mm (B) dimensions. The strips were air dried and blocked with 2% gelatin for 1 h at ambient temperature (28 $\pm$ 2°C). Excess gelatin was washed out thrice with TRIS buffer saline (TBS) containing 0.05% Tween 20 (TBST) for 10 min, each. The nitrocellulose strips were incubated with LLO antibody (as the case may be IgG / IgY) appropriately diluted with TBS of pH 7.2 for 1 h at ambient temperature. The strips were washed thrice with TBST for 10 min, each. The strips were incubated with secondary antibody conjugated with alkaline phosphatase enzyme (anti rabbit IgG / anti chick IgY) appropriately diluted in TBS of pH 9.5 at ambient temperature for 1 h. The strips were washed thrice with TBST of pH 9.5 for 10 min, each. The strips were incubated with BCIP-NBT substrate system at ambient temperature under dark conditions for 30 min. The nitrocellulose strips were observed for the appearance of purple colour. Nitrocellulose strips treated with no antigen served as control.

#### 4.2.2.9 Indirect ELISA

This ELISA protocol was optimized at ambient temperature. The antigen solution of appropriate concentration was prepared in TBS. The microtitre plates were coated with 100 ul of antigen solution in each well and incubated overnight at 4°C. Uncoated antigen solution was removed and the wells were blocked with 100 ul of 2% BSA in PBS. The microplates were incubated for 1 h and washed with TBST for 10 min, each. Antibody (IgG / IgY) appropriately diluted in TBS was added to each well and incubated

for 1 h. The plates were washed thrice with TBST for 10 min each after removing the uncoated antibody. Secondary antibody (anti rabbit IgG / anti chick IgY) conjugated with alkaline phosphatase enzyme was added to each well and incubated for 1 h. The uncoated antibody was removed and washed thrice with TBST for 10 min. Finally, the plates were treated with PNPP substrate in diethanolamine buffer for 30 min at ambient temperature in dark environment. The reaction of yellow colour formation was stopped by adding 3 M NaOH. The plates were read at 405 nm in ELISA Reader (Spectromax 340, Molecular Devices, USA).

**4.2.2.10 Colony blot**

The colony blot was performed according to the method described by Steinmetz (1992) with few modifications. The bacterial test cultures were grown for 24 h at 37°C. The nitrocellulose papers were laid on the surface of the agar plate with culture growth and allowed to adhere for 1 min. Nitrocellulose papers were then carefully removed, placed on the metal grid and exposed to hot steam for 5 min. Then the Nitrocellulose papers were blocked with 2% BSA for 30 min. The nitrocellulose strips were incubated with LLO antibody (IgG / IgY) appropriately diluted with TBS of pH 7.2 for 1 h at ambient temperature. The strips were washed thrice with TBST for 10 min, each. The strips were incubated with secondary antibody conjugated with alkaline phosphatase enzyme (anti rabbit IgG / anti chick IgY) appropriately diluted in TBS of pH 9.5 at ambient temperature for 1 h. The strips were washed thrice with TBST of pH 9.5 for 10 min, each. The strips were incubated with BCIP-NBT substrate system at ambient temperature under dark conditions for 30 min. The nitrocellulose strips were observed for the appearance of purple colour. Nitrocellulose strips treated with no antigen served as control.

#### 4.2.2.11 Antibody specificity

Antibody concentration was determined by following the protocol of Tsai and Yu (1997). The microtitre plates were coated with 100 ul of purified active fraction of LLO (100 ug, 10 ug, 1 ug, 100 ng, 10 ng,1 ng and 100 pg) and 100 ul of primary antibody (1: 1000, 1:10,000, 1:50,000, 1:1,00,000, 1:5,00,000 and 1:10,00,000). The assay was carried out as described above by reading OD at 405 nm in ELISA Reader.

#### 4.2.2.12 Detection of *Listeria* spp. in culture broth system

Cultures of *L*. *monocytogenes* Scott A, *L*. *monocytogenes* CFR 1338 and *L*. *innocua* Prd. 01 were grown in BHI broth for 24 h at 37°C. Culture broths were centrifuged at 8000 rpm for 20 min at 4°C and the resulting culture filtrates were diluted appropriately to get final dilutions of 1:0, 1:10, 1:100, 1:1000 and 1:10000. These dilutions of culture filtrates were subjected to indirect ELISA and dot blot assay as described previously. The cells obtained after centrifugation were used in colony blot assay as described previously.

#### 4.2.2.13 Detection of *L. monocytogenes* in milk

Cell suspension of a native food isolate of *L*. *monocytogenes* CFR 1338 was spiked into 10 ml quantities each of sterile skim milk to have individual population levels of $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ CFU/ml. These spiked milk samples were directly assayed for the presence of LLO by indirect ELISA and dot blot as described previously. Since liquid milk could not be separated into cells and supernatant very clearly without any additives, colony blot was not performed for the spiked milk samples.

#### 4.2.2.14 Use of immunoassay to detect *Listeria* in market samples of raw milk and raw vegetables

Ten samples each of raw milk and raw vegetables procured from the local market of the City of Mysore, India were enriched fro 24 h at 37°C in aliquots of BHI broth at a level of $10^{-1}$ dilution. The resulting culture supernatants obtained after centrifugation of culture broth at 8000 rpm for 20 min at 4°C (Refrigerated Centrifuge) were subjected to indirect ELISA and dot blot assay for the detection of *L. monocytogenes* in market samples.

#### 4.2.2.15 Detection of *L. monocytogenes* by the use of immunobeads

Immunobeads specific to *L. monocytogenes* were prepared for use in this protocol for detection of the organism.

*4.2.2.15.1 Preparation of Protein A beads*

Protein A coated Sepharose beads (Sigma Aldrich Co., Bangalore, India) were used to develop immunobeads for capturing *L. monocytogenes*. The Protein A beads were prepared as per the Manufacturer's instructions. Aliquots (6 ml) of the beads at a level of 1mg/ml were washed thrice with 20 mM potassium phosphate saline by centrifuging at 5000 rpm for 10 min at 4°C. After washing, the beads were diluted to the original volume with 20 mM PBS.

*4.2.2.15.2 Determination of antibody concentration*

The absorbance of different dilutions of prepared antibodies of LLO of *L. monocytogenes* i.e. IgG and IgY immunobeads was read at 280 nm in a UV-VIS Spectrophotometer (UV-160A, Shimadzu) and the antibody concentration was determined using the standard graph prepared with BSA.

*4.2.2.15.3 Binding of antibody to Protein A beads*

Aliquots of 0.1 ml of Protein A coated Sepharose beads to give a concentration of 1mg/ml were added to 0.3 ml quantities of different dilutions of antibody (IgG and IgY) and incubated for 1h 30 min with gentle shaking in a rotary shaker (Alpha Scientific Co., Bangalore, India). After incubation, beads were allowed to settle for 5 min and centrifuged at 5700 rpm for 20 min at 4°C. Pellet was washed thrice with 20 mM PBS and the volume made to 0.1 ml and used for immunocapture. Concentration of bound antibody was determined indirectly by measuring the unbound antibody present in the supernatant after centrifugation by reading the absorbance at 280 nm absorption and comparing with antibody solution before binding.

*4.2.2.15.4 Immunoseparation*

Antibody coated immunobeads in aliquots of 10 ul, 20 ul and 50 ul were added to 1 ml quantities of saline with individual cell populations of $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ CFU/ml of *L*. *monocytogenes* CFR 1338 prepared as described under **4.2.1.1** and incubated for 1 h 30 min at ambient temperature with gentle shaking. After incubation, the mixture was centrifuged at 1000 rpm for 3 min. The resulting pellet was washed thrice with PBS to remove the unbound bacteria. The bacteria bound to the beads were enumerated by plating them on listeria selective agar medium as described under **4.1.2.1**. The bacteria bound beads were also used in PCR, which would be described in the next experimental chapter of this thesis.

Similarly, immunoseparation was also performed by spiking of different cell population levels of *L*. *monocytogenes* CFR 1338 in aliquots of 1 ml of milk. The levels used were $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$ CFU/ml. The remaining procedure was the same as described previously.

### 4.2.3 RESULTS AND DISCUSSION

#### 4.2.3.1 Isolation and partial characterization of listeriolysin O (LLO)

The purpose of this experimental study was to purify the protein listeriolysin O (LLO) from *L*. *monocytogenes* culture for the purpose of raising antibody in rabbits and poultry. The anti-LLO produced was used in detection of *Listeria* spp. in culture broth and food matrix as well as market samples. Purified protein in higher amount was ideal to develop methods/assays to detect the pathogen. Listerial proteins like flagellar proteins, listeriolysin O, phospholipase C, metalloproteins, actin polymerizing proteins and several functionally unknown proteins have been in use over the years to detect *L. monocytogenes* (Bhunia, 1997). Listeriolysin O is a sentinel molecule for the detection of both disease caused and protection elicited by pathogenic *Listeria* (Berche et al.. 1990). LLO acts as a secretory indicator of the presence of *L*. *monocytogenes* in food.

In the present study, 13 haemolytic units per mg (HU/mg) of LLO were found to be present in *L*. *monocytogenes* Scott A and *L*. *monocytogenes* CFR 1338 in culture filtrates, when grown in BHI broth. Acetone precipitated extracellular protein showed increased haemolytic activity in both the cultures. Culture of *L*. *innocua* showed no haemolytic activity, even though it was containing same amount of protein in culture filtrate and the extracellular protein precipitated by acetone as shown in **Table 7**. In an earlier study, Perrisitus et al.. (1986) have estimated around 20-200 HU/ml of LLO in culture filtrate in conventional culture media which varied from strain to strain.

**Table 7** Estimation of listeriolysin O (Haemolytic activity) in cultures of *Listeria* spp.

| Culture | Culture filtrate | | Precipitated protein from culture filtrate | |
|---|---|---|---|---|
| | Protein Content (mg/ml) | Haemolytic activity (HU/mg, protein) | Protein Content (mg/ml) | Haemolytic activity (HU/mg, protein) |
| *L. monocytogenes* Scott A | 0.74 | 13.0 | 2.4 | 15.6 |
| *L. monocytogenes* CFR 1338 | 0.76 | 12.7 | 2.5 | 15.3 |
| *L. innocua* Prd. 01 | 0.72 | 0.0 | 2.3 | 0.0 |

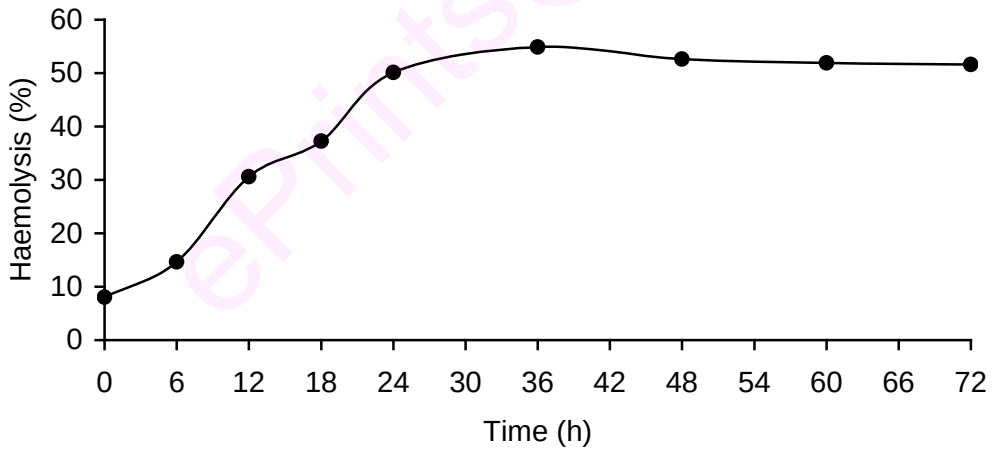



**Figure 2** Haemolytic activities associated with growth pattern of *L. monocytogenes* Scott A in BHI broth at different time intervals

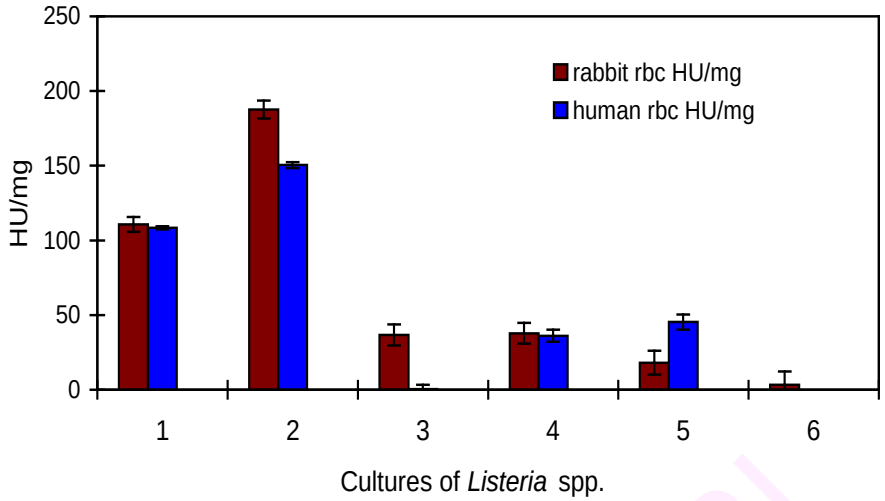



**Figure 3** Degree of haemolysis observed in culture supernatants (CS) and precipitated protein (PP) from the same supernatants in cultures of *Listeria* spp.
1, CS of *L*. *monocytogenes* Scott A; 2, CS of *L*. *monocytogenes* CFR 1338; 3, CS of *L*. *innocua* Prd. 01; 4, PP of *L*. *monocytogenes* Scott A; 5, PP of *L*. *monocytogenes* CFR 1338; 6, PP of *L*. *innocua* Prd. 01

Listeriolysin O is a member of family of thiol activated cytolysins which is secretory in nature (Palmer, 2001). Optimum quantity of LLO present in culture filtrate was obtained by subjecting the culture filtrates obtained at different periods of growth to haemolytic acitivty. The LLO production and secretion was optimum at 24 h of growth as could be seen in **Figure 2**. Hemolysis assay was carried out using the rabbit RBC, which is also generally compared with human RBC. In this study, no difference in haemolytic activity was observed with culture filtrates and extracellular proteins from *L*. *monocytogenes* Scott A and CFR 1338 (**Figure 3**). There was no appreciable activity being observed with the culture of *L*. *innocua* Prd. 01.

Purification of LLO was attempted in two step column chromatography. In the first step, the isolated extracellular protein obtained by acetone precipitation was

subjected to fractionation through Biogel P-100. The fractions in the range of 10-14 had the highest haemolytic activity. The LLO activity appeared to be not correlating with protein concentration, as could be seen in the peaks and its shoulders (**Figure 4**). Subsequently, lyophilized pooled active fractions from Biogel P-100 column were fractionated through Sephadex G-75 column. The highest haemolytic activity was observed in fractions 56-59, although significant peaks observed in the pattern did not yield appreciable HU (**Figure 5**). A summary of purification of LLO from *L. monocytogenes* Scott A is presented in **Table 8**.

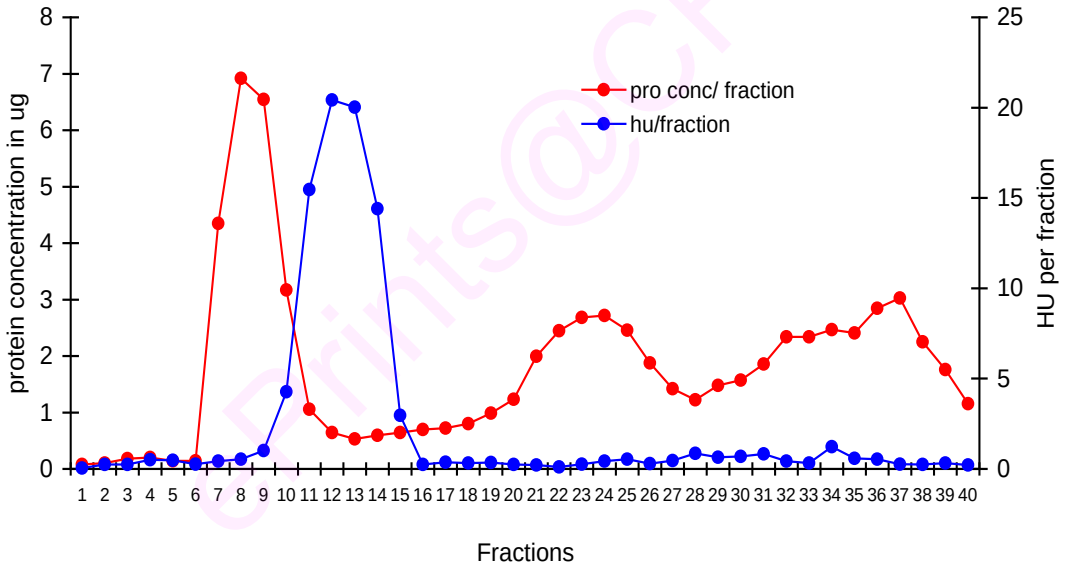



**Figure 4** Purification pattern of isolated listeriolysin O (LLO) from the culture of *L. monocytogenes* Scott A through Biogel P-100 column

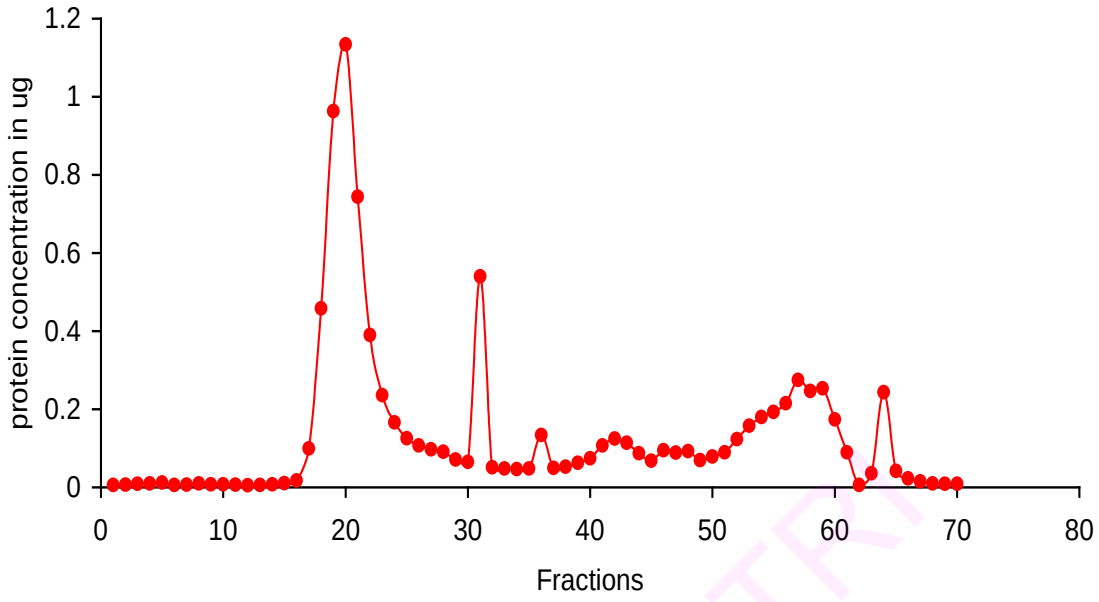



**Figure 5** Purification pattern in Sephadex G-75 column of pooled active fractions of LLO activity of *L. monocytogenes* Scott A obtained from Biogel P-100

**Table 8** Purification profile of listeriolysin O produced by *L. monocytogenes* Scott A

| Purification step | Volume (ml) | Total Protein content (mg) | Total haemolytic acitivty (HU x $10^4$) | Specific activity of haemolysis (HU/mg, protein) | Recovery (%) |
|---|---|---|---|---|---|
| Culture filtrate | 5000 | 3720 | 4.9 | 13.0 | 100.0 |
| Culture supernatant precipitated with acetone | 50 | 151 | 2.8 | 185.0 | 57.1 |
| Biogel P-100 fractions | 50 | 10 | 2.1 | 210.0 | 42.0 |
| Sephadex G-75 Fractions | 15 | 06 | 1.1 | 176.0 | 22.0 |

Geoffery et al. (1987) have purified LLO from culture filtrates of *L. monocytogenes* by concentrating and passing through thio-propyl sepharose 6B column, sephacryl S-200 column, Biogel P-100 column and fractogel HW–50 column. They could recover 1.3% of the total haemolytic activity that was 2.22 x $10^{-2}$ mg/litre. Yoshihava et al. (1993) have purified LLO from culture filtrates of *L. monocytogenes* by ammonium sulphate precipitation and passing through different columns to obtain a homogenous LLO for his study. In our study, LLO was purified from culture filtrate of *L. monocytogenes* by acetone precipitation. An approximately, 1.2mg/litre of partially purified LLO with 22% recovery of total haemolytic activity was achieved (**Table 8**).

Several researchers have purified LLO by culture filtrates by concentrating and passing through various purification protocols to obtain varied yields of LLO. Walton et al. (1999) purified LLO from Culture filtrates by concentrating through $CH_2$ spiral cartridge. Concentrate was passed through DEAE Sephacel and PD10 columns. About 13% of total haemolytic activity was recovered, which was 2.5 x$10^{-1}$ mg/litre. Low et al. (1992) had purified LLO from culture filtrates of *L. monocytogenes* by concentration, dialysis and fractionation through SP-cation exchange columns to obtain 6.25 x $10^{-1}$ mg/litre of pure LLO. Traub et al. (1995) have purified LLO from *L. monocytogenes* by hydroxy apatite adsorption and sepharose S ion-exchange columns to obtain 13.2 ug/litre. Baetz et al. (1995) have purified LLO from culture supernatants of *L. monocytogenes* in iron supplemented medium. Adsorption to Zetaprep sp100 capsule and performing protein liquid chromatography on Mono S column resulted in 52ug/litre of pure LLO.

Kayal et al. (1999) have purified LLO from EGD strain grown in RPMI-1640. The cell free culture supernatants was concentrated using 0.22 um filters and 30 kDa

miniplate. Subsequently, the concentrate was passed through DEAE–biogel A to homogeneity. Later, Nomura et al. (2002) had followed the above method with little modification. Lhopital et al. (1993) had purified LLO by preparative isoelectric focusing. The culture filtrates were concentrated by double filtration and passed twice through DEAE columns.

In recent times, several research teams carried out purification of recombinant LLO. Churchill et al. (2005) have purified recombinant LLO from *E*. *coli* with one step purification and obtained 2.5mg/litre, while Giammarini et al. (2003) have purified truncated LLO upto 4.5mg/litre. Darji et al. (1995) have purified recombinant LLO from *L*. *innocua* in culture supernatant by passing through Sepharose Q and Mono S columns to 1.6mg/litre with 63% recovery. Sbelius et al. (1999) have purified LLO from *L*. *innocua* harbouring plasmid PERL3. The culture supernatants were purified by concentration, batch absorption by Sepharose Q and eluted from Mono S HR5/5 column and dialyzed. Even though the production of LLO from these studies were more, the methods for purification from the culture filtrates after expression was quite extensive and time consuming and few have lacked the secretory signal resulting in truncated LLO and the constructs were less stable (Giammarini, 2003).

Molecular weight of LLO has been found to be 58 kDa (Walton, 1999; Churchill, 2005) and 60 kDa (Bhunia, 1997). The electrophoretically pure protein of our study was 58 kDa **(Figure 6)**. In the present study, the pH optima of purified active fraction of LLO showed activity only in acidic pH and no activity was observed in neutral pH (**Figure 7**). Similarly, the activity was predominant at 37°C incubation and not at other temperatures (**Figure 8**). The purified LLO was both, pH and temperature sensitive. A similar activity

profile has been shown in earlier studies (Palmer, 2001; Glomiski et al. 2003; Schuerch et al. 2005).

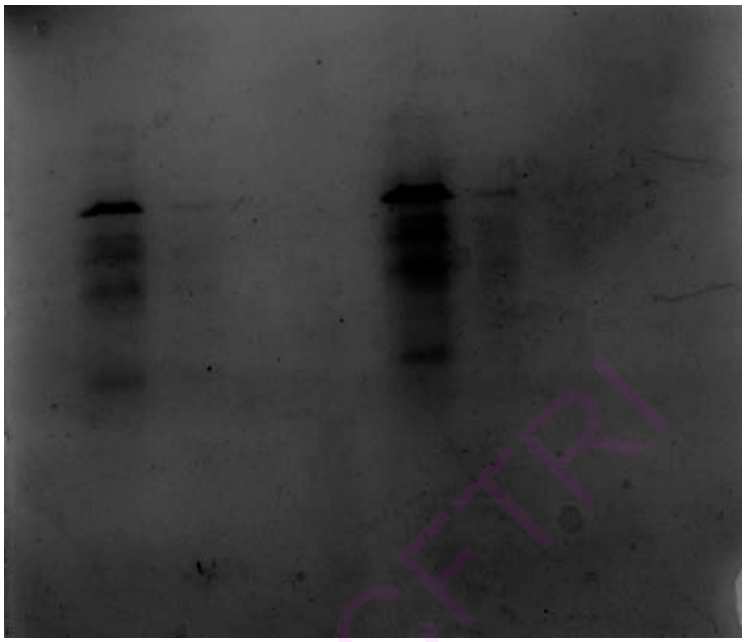


**Figure 6** SDS-PAGE of major fractions of haemolytic activity in the culture of *L. monocytogenes* Scott A obtained from Biogel P-100 column

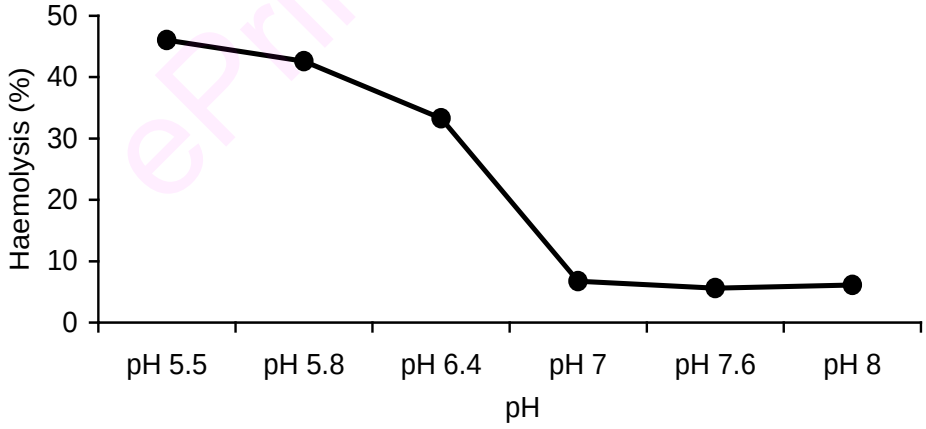



**Figure 7** Haemolytic activities of partially purified LLO of *L. monocytogenes* Scott A with human RBC at different pH levels

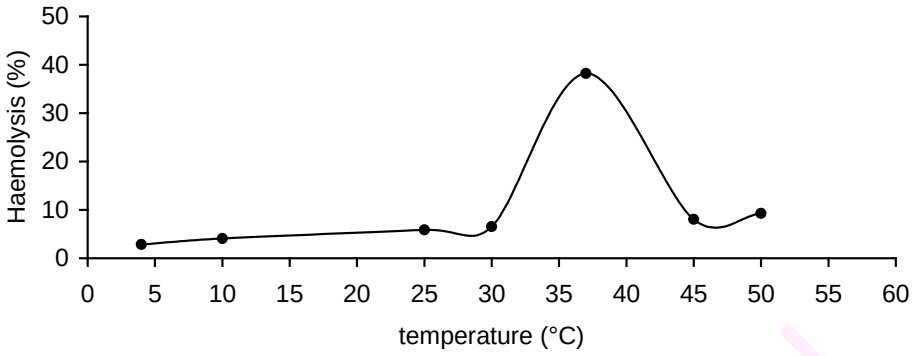



**Figure 8** Haemolytic activities of partially purified LLO of *L*. *monocytogenes* Scott A with human RBC at different temperatures

Darji et al. (1996) had used formaldehyde treated concentrated *L*. *monocytogenes* to develop several neutralizing monoclonal antibodies against listeriolysin and used it for characterization of recombinant LLO from *L*. *innocua*. Erdenlig et al. (1999) have used extracellular protein of *L*. *monocytogenes* to develop monoclonal antibodies in rabbit for the detection of *L*. *monocytogenes* in cat channel fish. Kreft et al. (1989) used purified LLO and iLO and produced antibodies to characterize the purified protein. Kathariou et al. (1990) developed polyclonal antibodies in rabbits to study transposon induced mutant strains by western blotting.

#### 4.2.3.2 Immunoassays based on LLO antibody production in rabbits and poultry

Purified LLO has been used to develop antibodies in sheep, dairy cattle and buffaloes. Low et al.(1992) used pure LLO as antigen to develop antibodies in lambs. Baetz et al. (1995) used purified LLO to raise antibodies in cows via intramammary

route, while Barbuddhe et al. (2002) had used purified LLO as antigen in buffaloes. Chicken immunoglobulin Y has been produced using *E*. *coli* by Sunwoo et al. (2006) to detect cultures of *E coli*. Flagellar proteins of *L*. *monocytogenes* were used for the production of IgY in poultry by Kim et al. (2005).

In the present study, the preparation of purified active fraction of LLO enabled the production high titered anti-LLO antibodies in rabbits (**Figure 9**) and poultry (**Figure 10**). The antibody produced in rabbit was purified from blood serum, while the antibody produced in poultry was purified from egg yolk. The production of antibody was higher in poultry as against that in rabbit. IgY at the dilution of 1:100000 was sufficient to detect 1 ng of purified LLO (**Figure 11**) as against that of 1:1000 dilution of rabbit IgG (**Figure 12**). The level of IgY in egg yolk was not steady to some extent, which was also reported by Kim. (2005). The fluctuation of IgY level was quite different from the normal IgG in blood of rabbit. Quantity of 100 ug of antigen was sufficient to produce high titre of yolk antibody compared to 400 ug of antigen for IgG production. Previous reports have also shown the production of abundant antibody in yolk, which can be a substitute for IgG in mammals (Tini et al. 2002).

ELISA has been the most common format used in immunodetection of pathogens. By this method, most pathogens were detected between $10^3$ to $10^5$ CFU/ml (De Boer and Beumer, 1999). Different types of ELISA techniques have shown to be a reliable screening technique to detect *Listeria* in food samples. Traub et al. (1995) had used the antibody raised against pure LLO for the detection of human listeriosis by direct ELISA. Barbuddhe et al. (1998) have used anti-LLO antibodies for the detection of experimental bovine listeriosis. Indirect ELISA technique has been used by Barbuddhe et al. (2002) for the detection of pathogenic *L*. *monocytogenes* in meat and milk using

the anti-LLO antibodies from buffalo. They could isolate 2.4% of *L. monocytogenes* from meat and 6.25% from milk samples. Similarly, Low et al. (1992) had used the antibody raised against purified LLO to diagnose the listeric infections in sheep by indirect ELISA method. Kim et al. (2005) had used the sandwhich ELISA technique to detect *L. monocytogenes* in food using the IgY antibody against flagellar antibodies. Torensma et al. (1993) have used whole cell ELISA for the identification of *Listeria* spp.

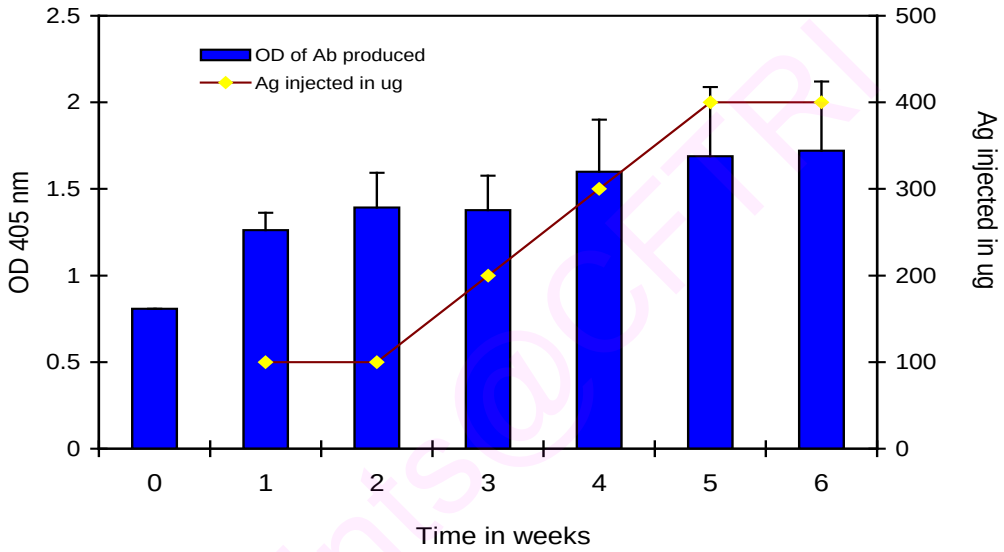



**Figure 9** Immunization and antibody production pattern of IgG in rabbits with purified active fraction of LLO

In our study, we have used indirect ELISA for the detection of *L. monocytogenes* in food samples. The antibody raised against purified active fraction of LLO in rabbit as well as Poultry were specific for the detection of *L. monocytogenes* Scott A and *L. monocytogenes* CFR 1338. However, at the same time, there also existed the possibility to detect *L. innocua* Prd. 01 to a certain extent (**Figures 13 and 14**). The cross reactivity with other Gram positive and negative bacteria was not very much evident. Cross reaction of anti-LLO antibody with other haemolytic bacteria has also been shown by Bhunia (1997).

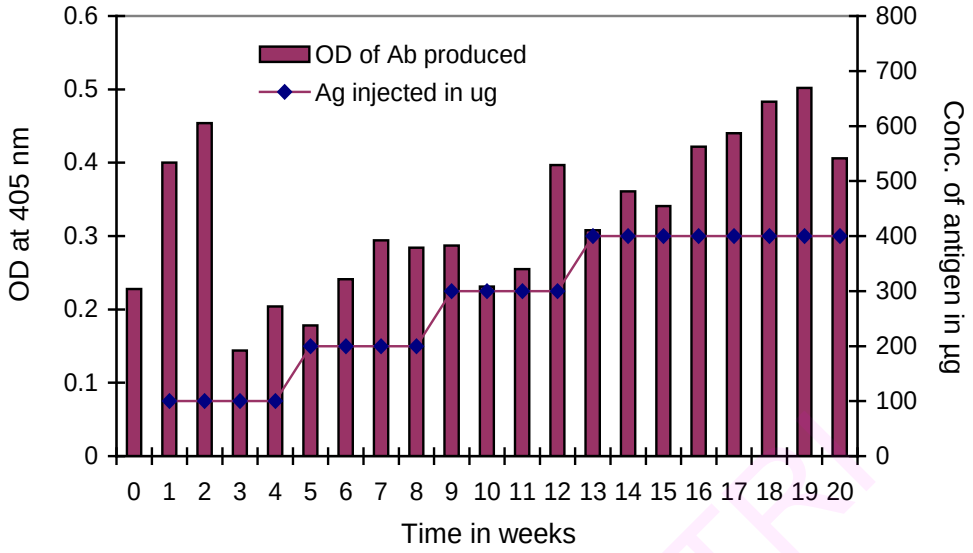



**Figure 10** Production pattern of IgY in poultry against purified active fraction of LLO

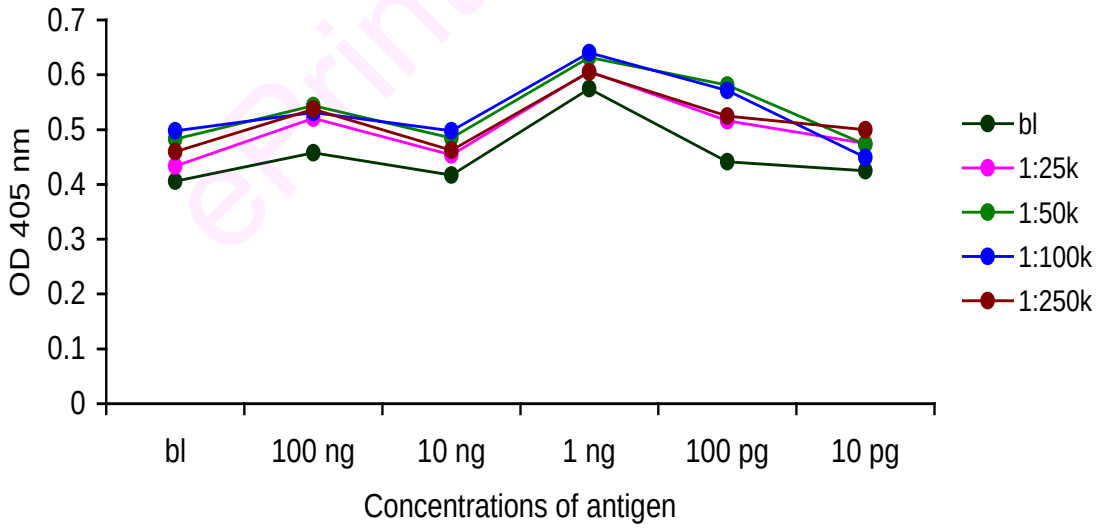



**Figure 11** Specificity of primary antibody (IgY) for purified active fraction of LLO

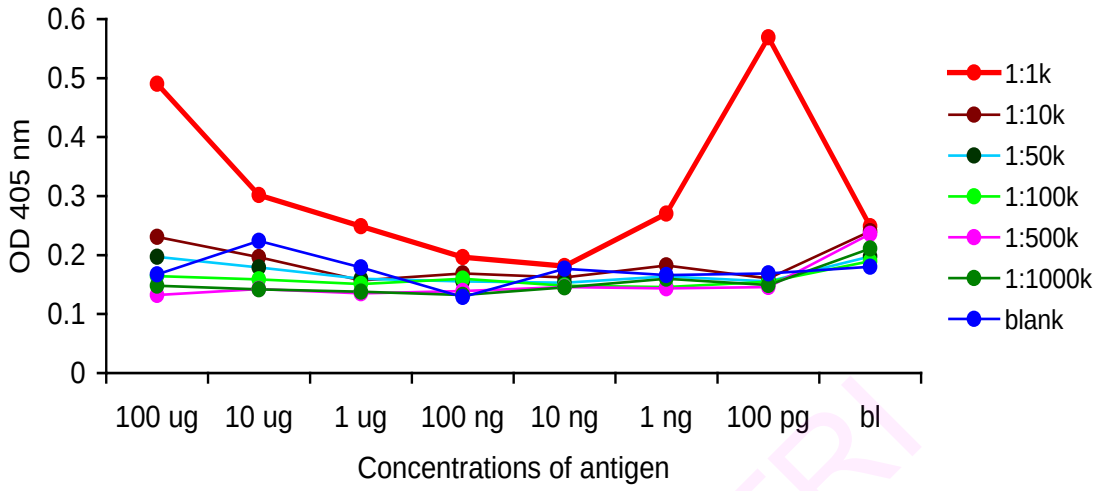



**Figure 12** Specificity of primary antibody (IgG) for purified active fraction of LLO

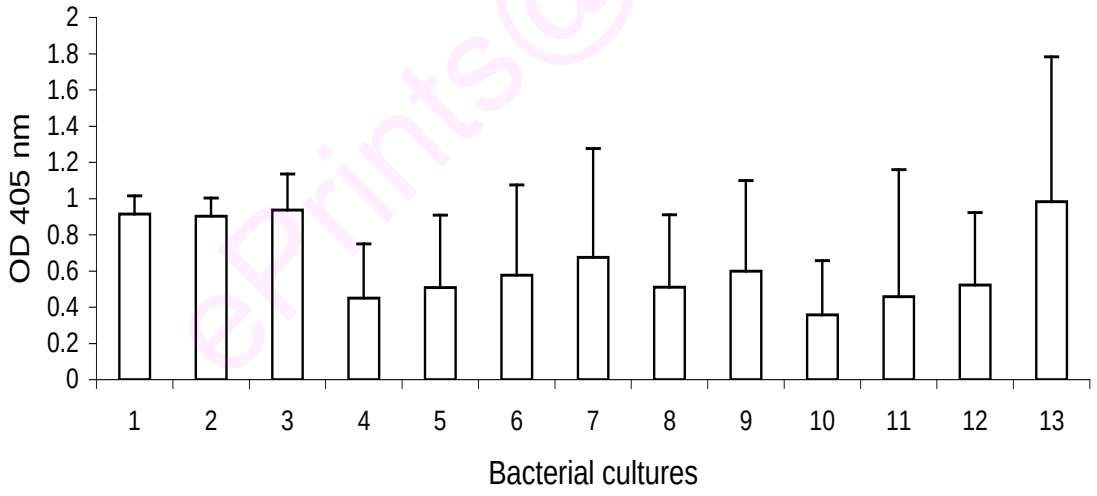



**Figure 13** Cross reactivity with other bacterial species for LLO antibody (IgG) raised in rabbits

1, *L*. *monocytogenes* Scott A; 2, *L*. *monocytogenes* CFR 1338; 3, *L*. *innocua* Prd. 01; 4, *L*. *grayi* Mng 03; 5, *L*. *grayi* subsp. *murrayi* Mng 04; 6, *L*. *seeligeri* Mng 02; 7, *L*. *ivanovii* Mng 01; 8, *E*. *coli* MTCC 118; 9, *Y*. *enterocolitica* MTCC 859; 10, *B*. *cereus* F4810; 11, *S*. *aureus* FRI 722; 12, Blank; 13, Partially purified LLO @ 100 pg

The same was also observed by visualization in Dot Blot (**Figure 15 A & B**) assay. Dot Blot method was used by Baetz et al. (1995) to detect *L*. *monocytogenes* in cows and humans. Lhophitol et al. (1993) have used Dot Blot technique for the detection of listeriosis in sheep. In our study, Dot Blot method was applied to detect *L*. *monocytogenes* in filtrates obtained from food samples.

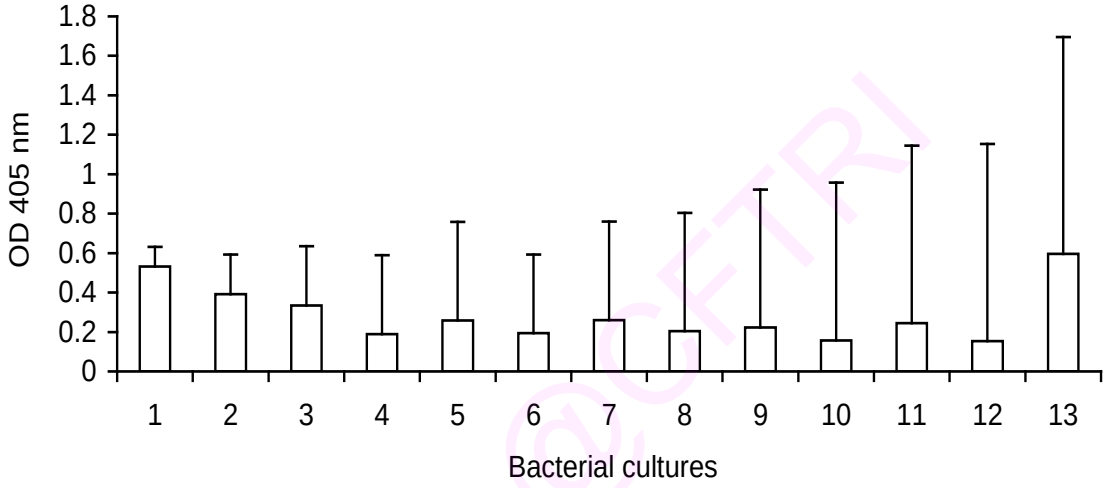



**Figure 14** Cross reactivity with other bacterial species for LLO antibody (IgY) raised in poultry

1, *L*. *monocytogenes* Scott A; 2, *L*. *monocytogenes* CFR 1338; 3, *L*. *innocua* Prd. 01; 4, *L*. *grayi* Mng 03; 5, *L*. *grayi* subsp. *murrayi* Mng 04; 6, *L*. *seeligeri* Mng 02; 7, *L*. *ivanovii* Mng 01; 8, *E*. *coli* MTCC 118; 9, *Y*. *enterocolitica* MTCC 859; 10, *B*. *cereus* F4810; 11, *S*. *aureus* FRI 722; 12, Blank; 13, Partially purified LLO at 100 pg

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The anti-LLO IgG as well as anti-LLO IgY could detect *L*. *monocytogenes* CFR 1338 to a level of $10^4$ CFU/ml in culture filtrates obtained after growth and appropriately diluted by indirect ELISA (**Figures 16 and 17**). In a similar way, the less sensitive Dot Blot method could detect $10^5$ CFU/ml

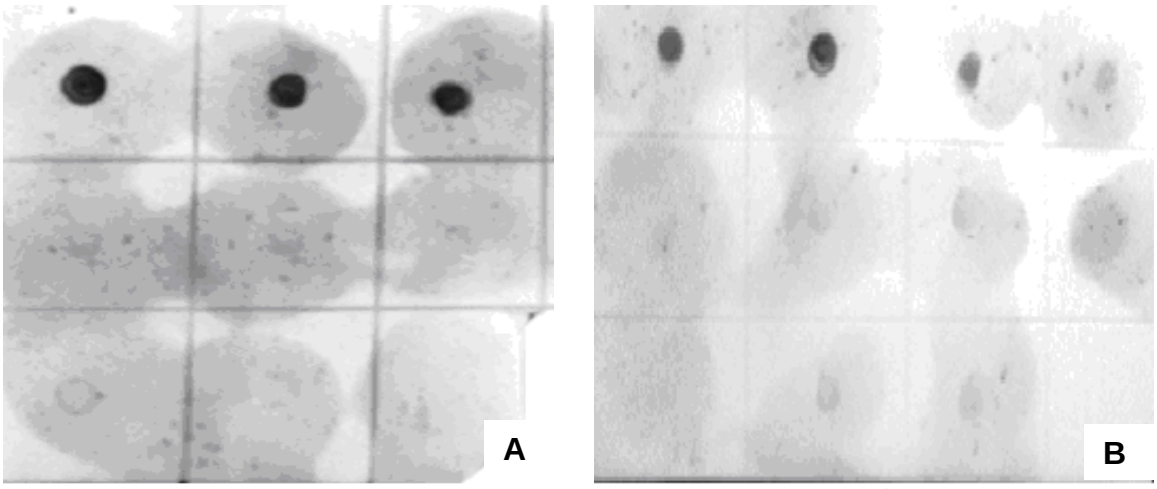


**Figure 15** Dot Blot showing the specificity of IgG [**A**] and IgY [**B**] towards different *Listeria* spp. and other Gram positive and negative bacteria

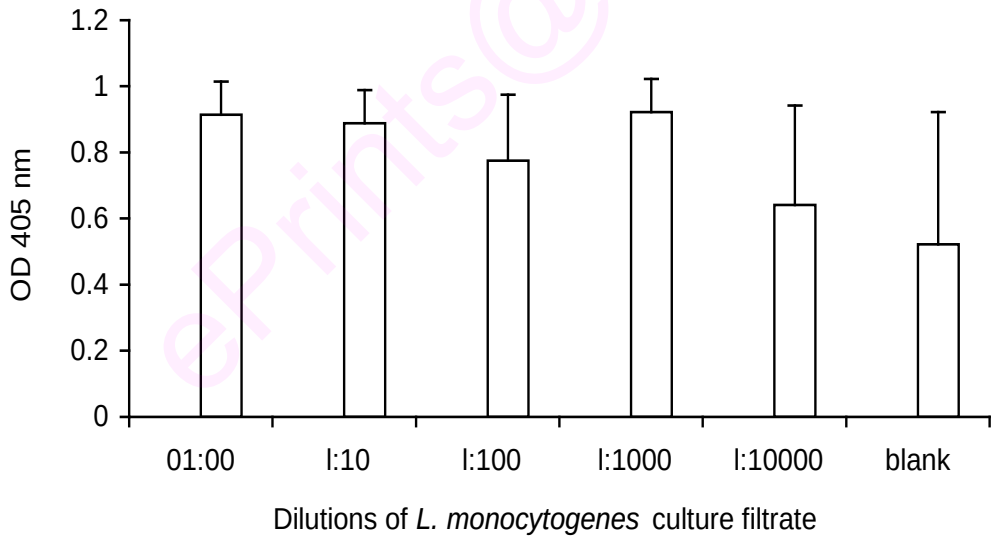


**Figure 16** Use of LLO antibody (IgG) in indirect ELISA to detect *L*. *monocytogenes* CFR 1338 in appropriate dilutions of cell-free culture filtrate

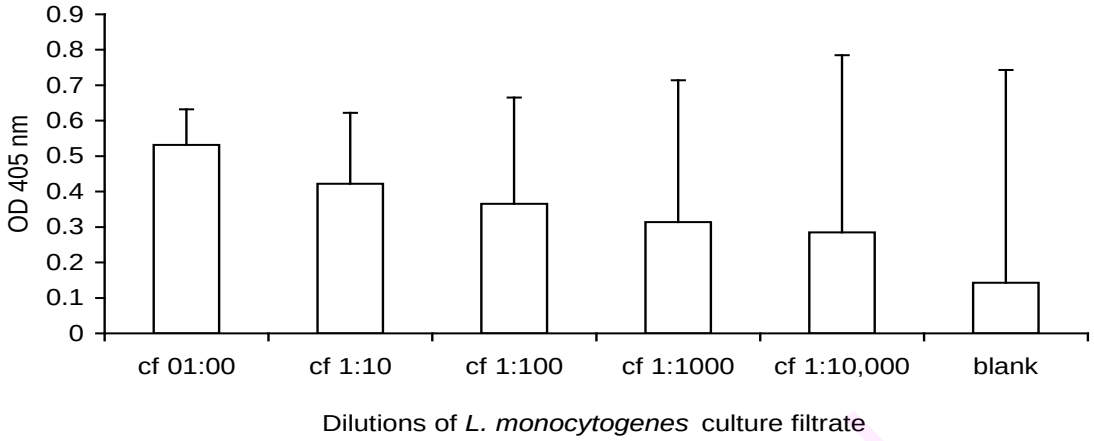


**Figure 17** Use of LLO antibody (IgY) in indirect ELISA to detect *L*. *monocytogenes* Scott A in appropriate dilutions of cell-free culture filtrate

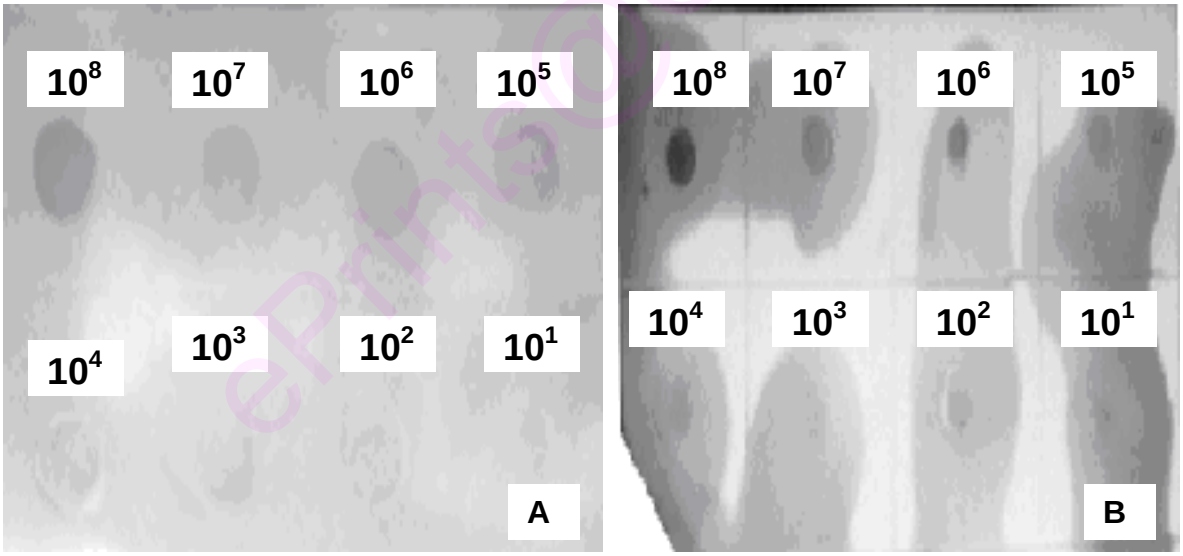


**Figure 18** Dot Blot with IgG [**A**] and IgY [**B**] for the detection of cell populations of *L*. *monocytogenes* CFR 1338 obtained by growing the culture in BHI broth

Considering the importance of detection methods for use in food systems, artificially inoculated skim milk with different cell populations of *L*. *monocytogenes* CFR 1338 was subjected to indirect ELISA, wherein with IgG and IgY the minimum detection

was to a level of $10^7$ CFU/ml (**Figures 19 and 20**). Similarly, the results obtained with Dot Blot assay are also presented for a comparative analysis (**Figure 21 A & B**).

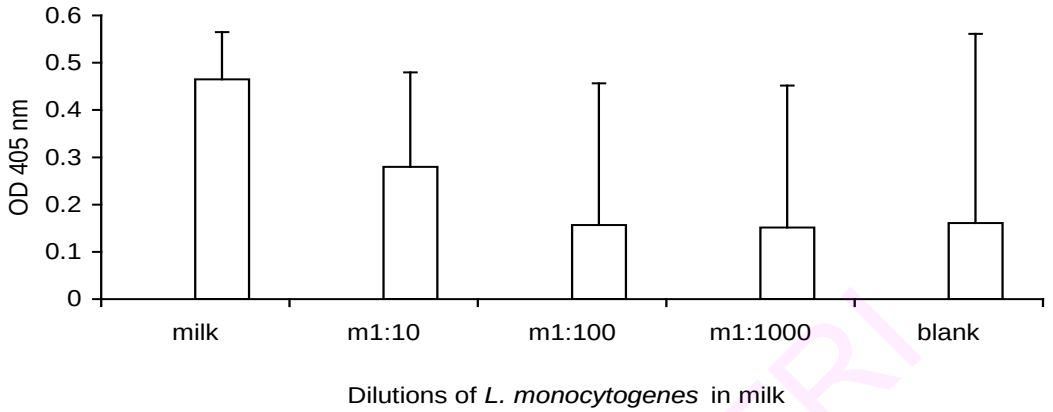


**Figure 19** Use of LLO antibody (IgG) in indirect ELISA to detect *L*. *monocytogenes* CFR 1338 spiked in fluid milk in appropriate dilutions of cell-free culture filtrate

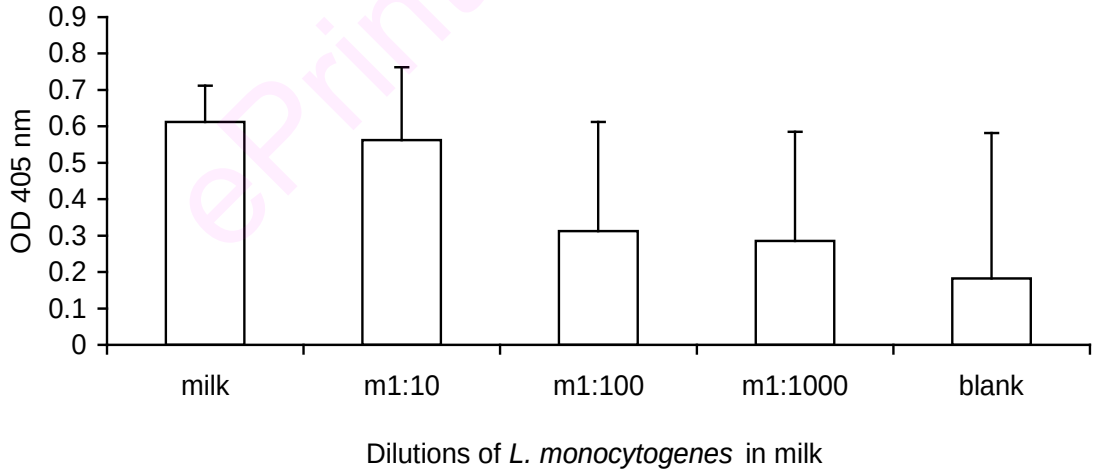


**Figure 20** Use of LLO antibody (IgY) in indirect ELISA to detect *L*. *monocytogenes* CFR 1338 spiked in fluid milk in appropriate dilutions of cell-free culture filtrate

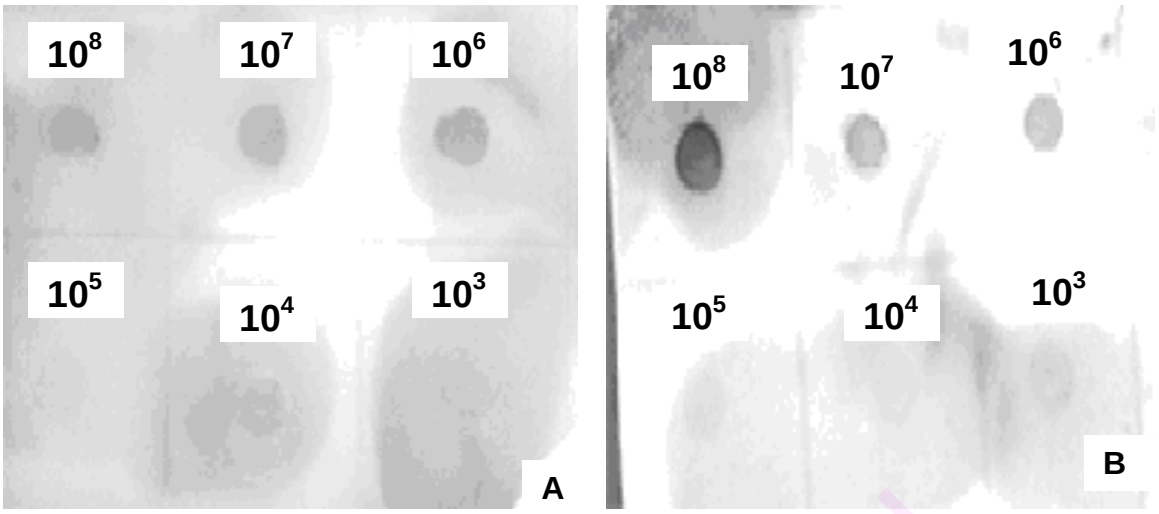


**Figure 21** Dot Blot with IgG [**A**] and IgY [**B**] for the detection of cell populations of *L*. *monocytogenes* CFR 1338 artificially spiked in skim milk and of devoid enrichment

#### 4.2.3.3 Immunoseparation approach for the detection of *L. monocytogenes*

The detection of pathogenic bacteria from a food matrix has been a difficult task in view of the presence of complex food constituents and the occurrence of pathogenic bacterial species like that of *L*. *monocytogenes* in very low numbers. As a means to enable an easier detection of low numbers of this organism, enrichment and immunoseparation methods are widely used. Enrichment method allows for the proliferation of cell numbers, while immunoseperation concentrates the pathogen making use of specific antibodies. Magnetic beads and protein A linked sepharose beads have been used to concentrate the pathogen to detectable levels in a few of the earlier studies (Hudson et al. 2001; Gray and Bhunia, 2005).

In the present study, protein A linked Sepharose beads were used to detect *L*. *monocytogenes* in food sample. By comparing with BSA, the concentration of anti-LLO IgG was found to be 8.2 mg protein/ml and anti-LLO IgY was 54.8 mg protein/ml.

Concentration of antibody bound to constant volume of 0.1 ml (0.1 mg) protein A coated Sepharose beads was determined indirectly by measuring the concentration of unbound antibody in the supernatant. There was no much difference in binding of IgG or IgY. The concentration of 0.5 mg each of IgG and IgY was found to be optimum for the preparation of immunobeads. In all, 30% of antibody was coated to the beads (**Figures 22 and 23**).

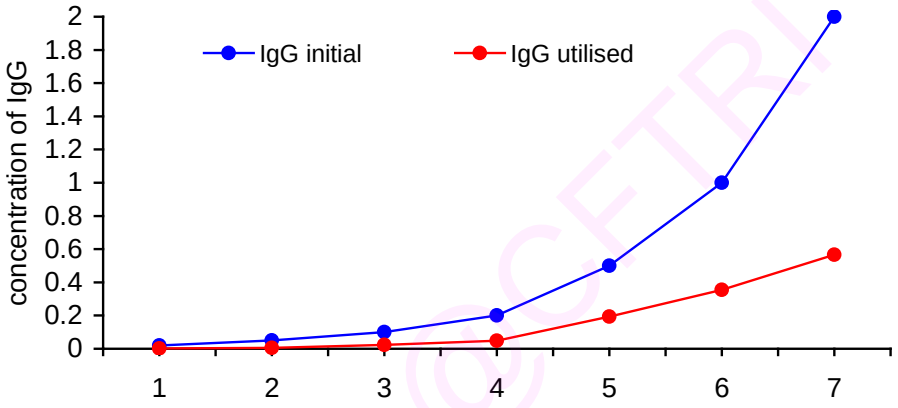


**Figure 22** Utilization pattern of IgG by protein A beads

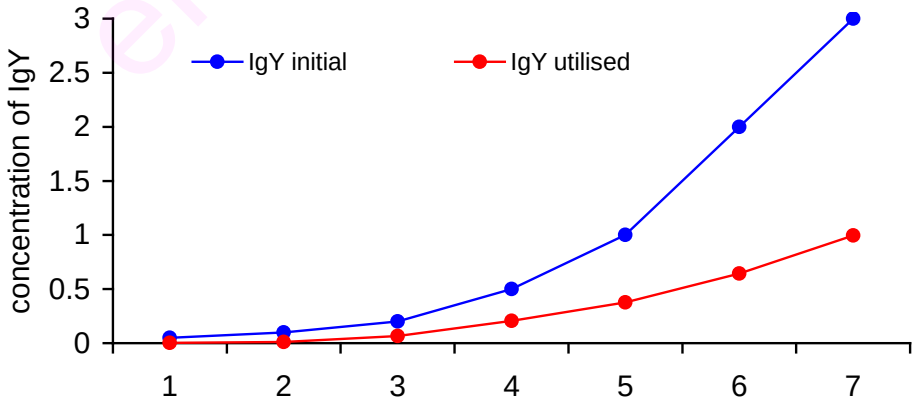


**Figure 23** Utilization pattern of IgY utilized by protein A beads

Gray et al. (2005) had used 0.01 – 10 mg of Pab –66 and Mab –CIIE9 for the preparation of immunobeads and could detect *L*. *monocytogenes* from meat samples. In the present experimental trials, immunoseparation method using IgG and IgY coated beads revealed no significant differences. Both the types of beads could detect *L*. *monocytogenes* to an extent of $10^4$ to $10^3$ CFU/ml, when cell suspensions were taken in saline (**Tables 9 and 10**).

**Table 9** Cell populations of *L*. *monocytogenes* CFR 1338 subjected to immunoseparation protocol with IgG coated immunobeads in saline

| Vol. of IgG beads | Different cell populations (CFU/ml) of *L*. *monocytogenes* in saline | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^9$ | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ | $10^2$ | $10^1$ |
| | Growth of *L*. *monocytogenes* on selective agar (CFU/ml) | | | | | | | | |
| 10 ul | OG | OG | 6310 ± 888 | 2963 ± 469 | 389 ± 19 | 56 ± 4 | NG | NG | NG |
| 20 ul | OG | OG | OG | 4142 ± 220 | 497 ± 72 | 44 ± 12 | 30 ± 6 | NG | NG |
| 50 ul | OG | OG | OG | OG | 624 ± 91 | 52 ± 1 | 43 ± 4 | NG | NG |
| 100 ul | OG | OG | OG | OG | 918 ± 87 | 225 ± 83 | 52 ± 6 | NG | NG |

OG, Over growth; NG, No growth

**Table 10** Cell populations of *L*. *monocytogenes* CFR 1338 subjected to immunoseparation protocol with IgY coated immunobeads in saline

| Vol. of IgG beads | Different cell populations (CFU/ml) of *L*. *monocytogenes* in saline | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^9$ | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ | $10^2$ | $10^1$ |
| | Growth of *L*. *monocytogenes* on selective agar (CFU/ml) | | | | | | | | |
| 10 ul | OG | OG | OG | 397 ± 15 | 408 ± 42 | 32 ± 13 | NG | NG | NG |
| 20 ul | OG | OG | OG | 584 ± 52 | 476 ± 98 | 40 ± 3 | NG | NG | NG |
| 50 ul | OG | OG | OG | 2042 ± 79 | 698 ± 51 | 80 ± 12 | NG | NG | NG |
| 100 ul | OG | OG | OG | OG | 856 ± 183 | 194 ± 127 | NG | NG | NG |

OG, Over growth; NG, No growth

The same iuumonseparation method could detect a minimum population of $10^5$ CFU/ml of *L*. *monocytogenes* CFR 1338 when artificially spiked into skim milk (**Tables 11 and 12**). These results could be very well achieved with 10 ul of immunobeads.

**Table 11** Cell populations of *L*. *monocytogenes* CFR 1338 subjected to immunoseparation protocol with IgG coated immunobeads in milk

| Volume of IgG beads | Different cell populations (CFU/ml) of *L*. *monocytogenes* in milk | | | | | |
|---|---|---|---|---|---|---|
| | $10^9$ | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ |
| | Growth of *L*. *monocytogenes* on selective agar (CFU/ml) | | | | | |
| 10 ul | OG | OG | OG | 825 ± 91 | NG | NG |
| 20 ul | OG | OG | OG | 836 ± 211 | NG | NG |
| 50 ul | OG | OG | OG | 990 ± 135 | 37 ± 17 | NG |

OG, Over growth; NG, No growth

**Table 12** Cell populations of *L*. *monocytogenes* CFR 1338 subjected to immunoseparation protocol with IgY coated immunobeads in milk

| Volume of IgG beads | Different cell populations (CFU/ml) of *L*. *monocytogenes* in milk | | | | | |
|---|---|---|---|---|---|---|
| | $10^9$ | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ |
| | Growth of *L*. *monocytogenes* on selective agar (CFU/ml) | | | | | |
| 10 ul | OG | OG | OG | 633 ± 91 | NG | NG |
| 20 ul | OG | OG | OG | 839 ± 71 | NG | NG |
| 50 ul | OG | OG | OG | 1089 ± 153 | 21 ± 11 | NG |

The study could project usefulness of developed immunological methods through the purification protocol for antibody production and optimize conditions to detect potential toxigenic / pathogenic traits in a native food isolate of *L*. *monocytogenes* CFR 1338.

## 4.3 MOLECULAR METHODS FOR THE DETECTION OF *LISTERIA* SPP.

In the present global scenario of no trade barriers for food products, the prime concern for food microbiologists /Food Regulatory Authorities has been the need to have detection methods for foodborne pathogenic bacterial species, which are rapid, reliable and specific towards targeting toxigenic/pathogenic traits in these organisms of health concern. The major drawback associated with the detection of *L*. *monocytogenes* has been the occurrence of this organism in very low numbers in contaminated food samples. The conventional enrichment-based methods are time consuming and are mostly genus targeted. Often, the final confirmation leads to the reporting of either false positive and/or false negative outcome. In this direction, there appears to have a promise in leading to established procedures to detect specific species/strains of *Listeria*, which harbour potential toxigenic traits. Although, commercial kits based on polymerase chain reaction have been in the use for detection of *L*. *monocytogenes*, the same have not found the approval of Food Regulatory Authorities in many of the countries around the globe. Often, the PCR methods established elsewhere have not been able to function due to aspects of microbial diversity of species/strains of the organism and their phenotypic traits as well as genetic relatedness.

In this background, the present experimental approach has attempted to design PCR primers targeting certain specific toxigenic traits known to prevail among species of Listeria and optimized conditions towards detecting Listeria spp. by uniplex PCR, multiple PCR, nested PCR and immuno PCR.

### 4.3.1 MATERIALS

All the glasswares used in the present investigation were either sterilized by autoclaving at 121°C for 20 min or by dry sterilization at 180°C for 4 h in a hot-air oven.

Gamma-irradiated sterile disposable petri plates, and dehydrated culture media procured from HiMedia laboratories, Mumbai, India. The media were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 min. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

The specific requisites in respect of PCR specific reagents and fine biochemicals such as Taq polymerase, dNTPs, base pair ladder molecular markers, proteinase K, Triton X, buffers, reagents, agarose, gel stain (ethidium bromide) used in this experimental study have been of molecular biology grade and obtained from Sigma-Aldrich, Bangalore, India; Bangalore Genei, Bangalore, India; and Sisco Research Laboratories, Mumbai, India.

**4.3.1.1 Bacterial cultures and inoculum preparation**

The cultures included were as follows:

i. Reference cultures of *L*. *monocytogenes* Scott A, *L*. *innocua* Prd. 01, *L*. *ivanovii* Mng 01, and *L*. *seeligeri* Mng 02, the details of which were documented under **4.2.1.1**.

ii. Native food isolates of *L*. *monocytogenes* CFR 1338, *L*. *ivanovii* CFR 1344 and *L*. *grayi* subsp. *grayi* CFR 1352, which were isolated from samples of milk-based traditional sweet, raw meat and raw vegetable, respectively, in this programme (Results of **4.1**). The cultures were maintained at 4°C on brain heart infusion (BHI) agar slants and subcultured at 15 d intervals. The cultures were propagated twice in BHI broth at 37°C prior to use in experiment.

Prior to use in experiments, the individual cultures of *L*. *monocytogenes* Scott A and *L*. *monocytogenes* CFR 1338 were successively propagated twice in BHI broth for 24 h at 37°C in an orbital shaker incubator (Alpha Scientific Co., Bangalore, India) at 140 rpm. Cell suspensions of these cultures were individually prepared from 20 h old BHI culture broth (grown under conditions previously described) by centrifugation at 8000 rpm for 20 min at 4°C (Superspin R-V/$F_M$, Plasto Crafts, Mumbai, India). The harvested cells were resuspended in sterile 10 ml aliquots of 0.85% saline and stored in sterile screw-capped tubes at 4°C until further use.

**4.3.1.2 Diluent**

The diluent used was 0.85% normal saline, which was dispensed in requisite quantities in suitable glass containers and autoclaved.

**4.3.1.3 Brain heart infusion (BHI) broth and agar**

This was prepared as described under **4.1.1.5.2** (**A**) and used in this study.

**4.3.1.4 Skim milk**

This was prepared by as described under **4.2.1.4** and used in this study.

**4.2.1.5 PCR primers**

Oligonucleotide primers were designed for haemolysin in general for pathogenic *Listeria* spp. and listeriolysin O, ivanolysin and seeligerolysin specific to toxigenic species of *L*. *monocytogenes*, *L*. *ivanovii* and *L*. *seeligeri*, respectively, based on the GenBank sequences and using the software programme of Dialign 2: a multiple sequence alignment. A common forward primer of haemolysin and different reverse primers were used to detect *Listeria* spp. in multiplex and nested PCR. The nucleotide

sequences of the primers designed are shown in **Table 13**. The synthesized primers were obtained from a commercial company (Sigma Aldrich, Bangalore, India).

**Table 13.** Nucleotide sequence of specific primers and PCR conditions used in the detection of *L. monocytogenes* and other *Listeria* spp.

| Target gene | Sequence / PCR conditions | Amplicon Size (bp) |
|---|---|---|
| Haemolysin | 5′ GTTAATGAACCTACAAGHCCTT 3′<br>5′ TYGCRTTHCCTGGCAAATAGAT 3′<br>94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 10′; (35 cycles) | 622 |
| Listeriolysin O | 5′ GTTAATGAACCTACAAGHCCTT 3′<br>5′ TGGCAAGAGATGTTGAATTGA 3′<br>94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 10′; (35 cycles) | 470 |
| Ivanolysin | 5′ GTTAATGAACCTACAAGHCCTT 3’<br>5’ ACGTTCAACAACTTCATTTCCA 3’<br>94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 10′; (35 cycles) | 520 |
| Seeligerolysin | 5′ GTTAATGAACCTACAAGHCCTT 3’<br>5’ GCGTTCTCTATCATAAGTGGA 3’<br>94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 10′; (35 cycles) | 250 |

Haemolysin is for the pathogenic species of Listeria

Other primers are specieis specific (pathogenic)

Forward primer of haemolysin and different reverse primers were used to detect *Listeria* spp. in multiplex and nested PCR

### 4.3.2 METHODOLOGY

#### 4.3.2.1 Isolation of DNA from isolates of *Listeria* spp.

Isolation of DNA from cultures of *Listeria* spp. was performed following the established protocols described by Sambrook and Russel (2001) with a few modifications. Test cultures (**4.3.1.1**) were grown individually in BHI broth for 14 h at 37°C in an orbital shaker incubator (Alpha Labs., Bangalore, India) at 140 rpm. Aliquots of this culture were plated on BHI agar to obtain single and discrete colonies. Single and well-isolated colonies were once again grown overnight in BHI broth for 14 h at 37°C at 140 rpm. Aliquots of culture broth in 1.0 ml were centrifuged at 8000 rpm for 20 min at 4°C (Refrigerated Centrifuge). The resulting pellet was suspended in 250 µl of lysis buffer consisting of 0.1 M Tris-HCl of pH 8.0 with 2% Triton-X and 2 mg/ml of lysozyme and incubated for 30 min at 37°C. To this was later added 25 µl of proteinase K (10mg/ml) incubated for 2 h at 56°C. After completing the incubation period, 275 µl of Phenol : Chloroform : Isoamylalcohol : Water (in the ratio of 25:24:1:50) was added and centrifuged at 10000 rpm for 10 min at 4°C. The resulting supernatant was collected and 275 µl of Isopropylalcohol with 150 ul of 5M NaCl was added slowly and centrifuged at 10000 rpm for 10min at 4°C. The resulting pellet was washed with 70% alcohol, air-dried, suspended in 10 ul of TE buffer and stored in sterile screw-capped tubes at –20°C till further use in the experimental trials. As a means to make sure about the preparation of template DNA, quantification of DNA in the finally prepared template was undertaken by recording the absorbance at 260 and 280 nm in a UV-VIS Spectrophotometer (UV 160-A, Shimadzu) and calculating the ratio.

**4.3.2.2 PCR amplification of haemolysin, listeriolysin O, ivanolysin and seeligerolysin among isolates of *Listeria* spp.**

PCR was performed for the detection of specific target genes in the cultures of *L. monocytogenes* Scott A and CFR 1338; *L. ivanovii* Mng 01 and CFR 1344 and *L. seeligeri* Mng 02.

The Reaction components for PCR amplification included the following:

i. Template DNA
ii. Gene specific primers (Table 13)
iii. *Taq* DNA polymerase (3 units/μl, Bangalore Genei, India)
iv. 10X Reaction Buffer: 50 mM Tris-HCl of pH 8.3 and 2.5 mM $MgCl_2$
v. Nuclease-free water
vi. dNTP mix (10mM of each dNTP)

For a reaction volume of 25 μl, the following reaction components were combined in a thin-walled 0.2 ml PCR reaction tube.

| Components | Volume (μl) | Final concentration |
|---|---|---|
| Nuclease-free water | 16.7 | |
| 10 X Reaction Buffer | 2.5 | 1 X |
| dNTP mix (10 mM each) | 0.5 | 0.2 mM each |
| *Taq* DNA polymerase (3U/ μl) | 0.2 | 0.03 U/ μl |
| Gene specific Primer F | 0.5 | 0.2 μM |
| Gene specific Primer R | 0.5 | 0.2 μM |
| Template | 4.0 | |

The contents of the tube were mixed by a brief spin in a microcentrifuge. PCR amplification was performed in an automated DNA Thermal Cycler (Eppendorf, Master Cycler, Cedex, France) following the PCR conditions as detailed in Table 13.

**4.3.2.3 Analysis of PCR products by agarose gel electrophoresis**

The resultant PCR product was analyzed by agarose gel electrophoresis using the following materials (Sambrook and Russel, 2001):

i. Agarose
ii. TAE buffer (50 X)
iii. 24.2 g Tris base, 5.71 ml of glacial acetic acid and 10 ml of 0.5 M EDTA (pH 8.0) was added to 75 ml of Milli Q water. The pH of the buffer was adjusted to 7.2 and the final volume was brought to 100 ml with Milli Q water. The buffer was sterilized by autoclaving and stored at ambient temperature.
iv. Orange loading dye (6X)
v. Gel running boat
vi. Submarine gel electrophoretic apparatus and power supply
vii. Ethidium bromide stock solution (10 mg/ ml)
viii. 10 mg of ethidium bromide was dissolved in 1 ml of Milli Q water. The solution was dispensed in a microcentrifuge tube and stored in dark at 4°C.

The electrophoresis was performed as follows:

i. The gel running boat was sealed with an adhesive tape and the comb was installed at the appropriate position for the wells.
ii. Agarose in 2 g quantity was added to 100 ml of 1 X TAE buffer. The mixture was heated on a hot plate to dissolve agarose.
iii. The solution was cooled to 50°C and poured into the sealed boat.
iv. The gel was allowed to set. The comb and the adhesive tape were removed and the gel was placed in the electrophoresis tank.
v. Sufficient volume of 1X TAE buffer was added to the tank to cover the surface of the gel.

vi. A 10 μl aliquot of each sample was loaded after mixing with 2 μl of the loading dye.

vii. A molecular size marker prepared according to the manufacturer's instructions, was loaded along with the samples.

viii. Electrophoresis was carried out at 100 volts until the dye reached 3/4$^{th}$ of the gel.

ix. The gel was removed from the tank and stained by soaking in a solution of 0.5 μg/ml ethidium bromide for 30 min at ambient temperature.

x. The gel was destained in distilled water for 10 min, placed on a UV-transilluminator and the image was documented in Gel Documentation System (Vilber Lourmat, France).

#### 4.3.2.4 Detection of cell populations of *L. monocytogenes* in saline and skim milk

In these experimental trials, aliquots in 1 ml of cell suspension of *L. monocytogenes* CFR 1338 in normal saline and skim milk, respectively, were prepared from the stock cell suspension to get individual populations of $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ CFU/ml. The aliquots of saline and milk with different cell populations were subjected to isolation of template DNA and PCR as described previously (**4.3.2.1 to 4.3.2.3**) for the targeted gene of listeriolysin O.

#### 4.3.2.5 Nested PCR for haemolysin and listeriolysin genes in the food isolate of *L. monocytogenes* CFR 1338

In this trial, template DNA of *L. monocytogenes* CFR 1338 grown in BHI broth was prepared as described under **4.3.1.1** and subjected to uniplex PCR with haemolysin primers as described previously. In the next step, the resultant PCR product from the above was used to perform PCR with listeriolysin O primers (Table 13). The reaction

components were the same as described under **4.3.2.2** and the reaction volume was as follows:

For a reaction volume of 25 μl, the following reaction components were combined in a thin-walled 0.2 ml PCR reaction tube.

| Components | Volume (μl) | Final concentration |
|---|---|---|
| Nuclease-free water | 18.7 | |
| 10 X Reaction Buffer | 2.5 | 1 X |
| dNTP mix (10 mM each) | 0.5 | 0.2 mM each |
| *Taq* DNA polymerase (3U/ μl) | 0.2 | 0.03 U/ μl |
| Gene specific Primer F | 0.5 | 0.2 μM |
| Gene specific Primer R | 0.5 | 0.2 μM |
| Template | 0.2 | |

The contents of the tube were mixed by a brief spin in a microcentrifuge. PCR amplification was performed in an automated DNA Thermal Cycler (Eppendorf, Master Cycler, Cedex, France) and analysis of PCR amplified products was carried out as described under **4.3.2.3**.

#### 4.3.2.6 Multiplex PCR for haemolysin and listeriolysin genes in the food isolate of *L. monocytogenes* CFR 1338

In this trial, template DNA from different cell populations ($10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ CFU/ml) of *L. monocytogenes* CFR 1338 in 1.0 ml aliquots of saline was performed as described under **4.3.2.1** and used to perform multiplex PCR using forward and reverse primers of haemolysin and reverse primer of listeriolysin O. The reaction components for PCR amplification were the same as described under **4.3.2.2**. The reaction volume is as follows:

For a reaction volume of 25 μl, the following reaction components were combined in a thin-walled 0.2 ml PCR reaction tube.

| Components | Volume (μl) | Final concentration |
|---|---|---|
| Nuclease-free water | 17.7 | |
| 10 X Reaction Buffer | 2.5 | 1 X |
| dNTP mix (10 mM each) | 0.5 | 0.2 mM each |
| *Taq* DNA polymerase (3U/ μl) | 0.2 | 0.03 U/ μl |
| Gene specific Primer F | 1.0 | 0.2 μM |
| Gene specific Primer R1 & R2 | 0.5 & 0.5 | 0.2 μM |
| Template | 2.0 | |

The contents of the tube were mixed by a brief spin in a microcentrifuge. PCR amplification was performed in an automated DNA Thermal Cycler (Eppendorf, Master Cycler, Cedex, France) and analysis of PCR amplified products was carried out as described under **4.3.2.3**.

**4.3.2.7 Single colony PCR and immuno PCR for *L. monocytogenes* CFR 1338**

In this experimental approach, from the BHI agar grown plates with culture of L. Monocytogenes CFR 1338,a single and discrete colony (as described under **4.3.2.1**) was picked up without any cross contamination and directly transferred to PCR tube and subjected to PCR with listeriolysin primers as described under **4.3.2.2** and **4.3.2.3** without the step of preparing template DNA.

In a similar approach, immuno PCR was performed, wherein individual cell populations of $10^1$, $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, $10^7$, $10^8$ CFU/ml of *L. monocytogenes* CFR 1338 in 1.0 ml aliquots of saline were taken in 1.5 ml microcentrifuge tubes. To these individual tubes were added 50 μl of IgG / IgY coated immunobeads (prepared as

described under **4.2.2.15**) and incubated for 1 h at ambient temperature. After the incubation period, the contents of tubes were centrifuged at 2000 rpm for 10 min at 4°C. The resulting pellet was washed twice with PBS and aliquots of 2 µl of pellet was used directly to perform PCR with listeriolysin primers as described previously, without the step of preparing template DNA.

### 4.2.3 RESULTS AND DISCUSSION

#### 4.2.3.1 PCR detection of toxigenic / pathogenic traits in *Listeria* spp.

This experimental study has attempted to focus on the optimization and adaptability of PCR detection methods for the occurrence of toxigenic / pathogenic traits among cultures of *L*. *monocytogenes* and related species. A few selected pathogenic traits were focused ad primers were designed specifically to detect the same with emphasis on specificity and reliability. The study included reference cultures and a few native food isolates from this study and detection protocols were undertaken in pure culture broth system and skim milk by uniplex, multiplex and immmuno PCRs.

The DNA was isolated by from different *Listeria* spp. by the method described by Sambrook and Russel (2001) with little modifications. This protocol was simple to use and had high sensitivity and reproducibility. The purity and integrity of isolated DNA was examined by agarose gel electrophoresis. The concentration of DNA was found to be 0.03-0.4 ug/ml of bacteria.

The genus *Listeria* includes the two pathogenic species *L. monocytogenes* and *L*. *ivanovii* and the four apparently apathogenic species *L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi.* Pathogenicity of the former two species is enabled by an approximately 9 kb virulence gene cluster which is also present in a modified form in *L.*

*seeligeri* (Hain, 2006), hence the primers were designed for haemolysin gene which is present in all the pathogenic *Listeria* spp and Listeriolysin O, ivanolysin and seeligerolysin for the individual *Listeria* spp.

Primers designed for haemolysin could amplify all the pathogenic *Listeria* spp. to an amplicon of 622 bp (**Figure 24**). Listeriolysin primers amplified 470 bp amplicon from *L. monocytogenes* (**Figure 25**), ivanolysin amplified 520 bp amplicon from *L. ivanovii* (**Figure 26**) and seeligerolysin amplified 250 bp amplicon from *L. seeligeri* (**Figure 27**). The native isolate *L*. *monocytogenes* CFR 1338 produced positive amplicon along with the reference culture of *L*. *monocytogenes* Scott A for the sequence specific for haemolysin and listeriolysin. The native isolate of *L. ivanovii* CFR 1344 produced positive amplicon along with reference culture of *L. ivanovii Mng* 01 for the sequence specific to ivanolysin and haemolysin.

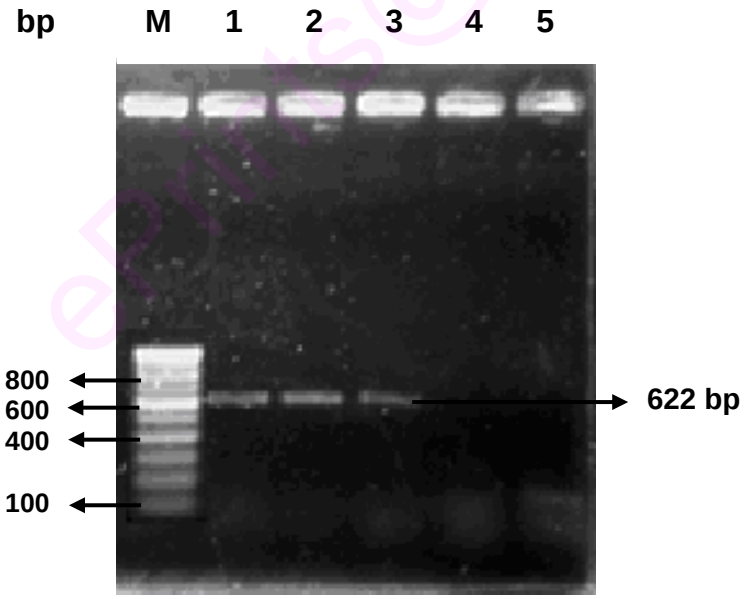


**Figure 24** Agarose gel electrophoretic pattern of PCR products showing amplicons with haemolysin primers in cultures of *Listeria spp.*

Lane **M**, Marker of 100 bp plus ladder; Lanes **1**, *L. monocytogenes* Scott A; **2**, *L. ivanovii* Mng 01; **3**, *L. seeligeri* Mng 02; **4**, *L*. *innocua* Prd. 01; *L*. *grayi* subsp. *grayi* Mng 03

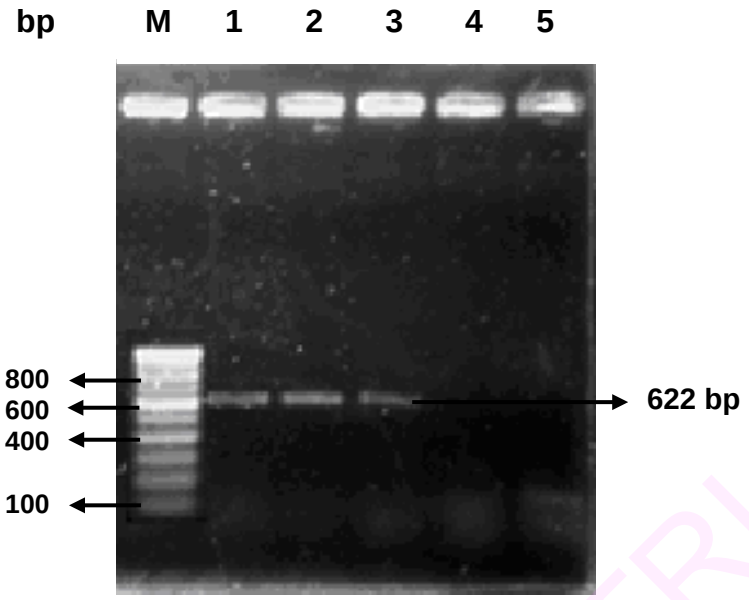


**Figure 25** Agarose gel electrophoretic pattern of PCR products showing amplicons with haemolysin primers in cultures of *Listeria spp.*

Lane **M**, Marker of 100 bp plus ladder; Lanes **1**, *L. monocytogenes* Scott A; **2**, *L. ivanovii* Mng 01; **3**, *L. seeligeri* Mng 02; **4**, *L*. *innocua* Prd. 01; *L*. *grayi* subsp. *grayi* Mng 03

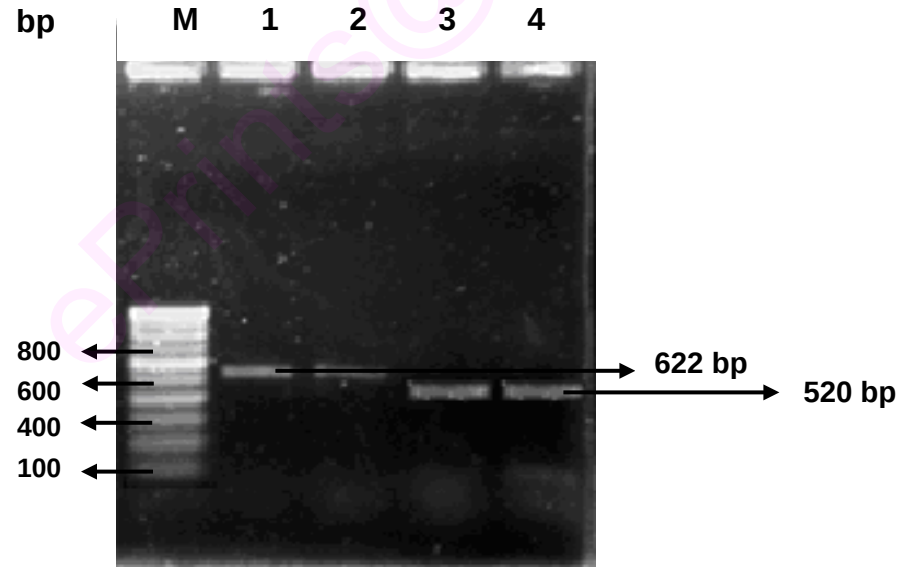


**Figure 26** Agarose gel electrophoretic pattern of PCR products showing amplicons with haemolysin and ivanolysin primers in cultures of *Listeria spp.*

Lane **M**, Marker of 100 bp plus ladder; Lanes **1**, *L. ivanovii* Mng 01; and **2**, *L. ivanovii* CFR 1344 for haemolysin; **3**, *L. ivanovii* Mng 01; and **4**, *L. ivanovii* CFR 1344 for ivanolysin

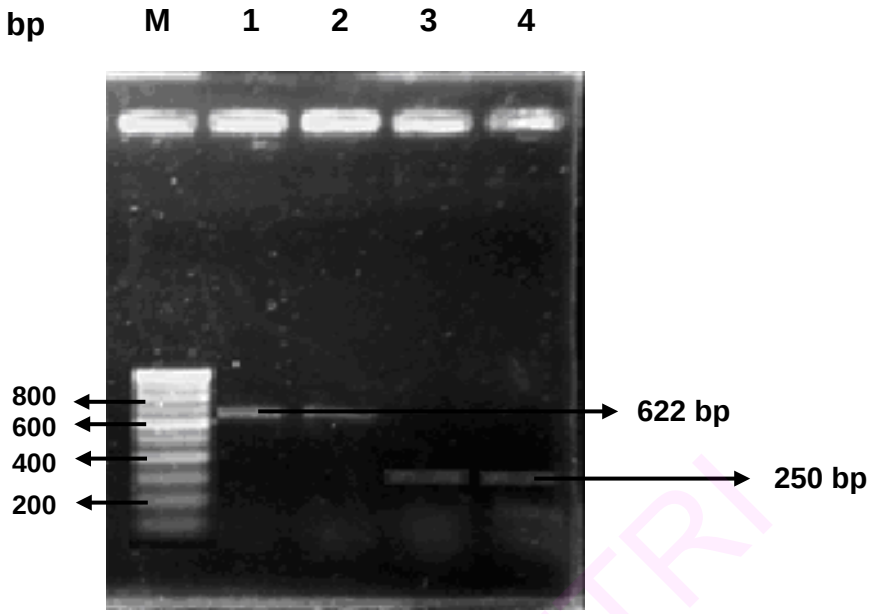


**Figure 27** Agarose gel electrophoretic pattern of PCR products showing amplicons with haemolysin and seeligerolysin primers in the culture of *L. seeligeri* Mng 02
Lane **M**, Marker of 100 bp plus ladder; Lanes **1 & 2**, Positive for haemolysin; **3 & 4**, Positive for seeligerolysin

Listeriolysin (*hly*A) gene has been used by Norton et al. (2001) and Thimothe et al. (2004) for the detection of *L. monocytogenes* by uniplex PCR in smoked salmon. Isolation of *L. monocytogenes* from buffaloes with reproductive disorders was confirmed by polymerase chain reaction using hlyA gene by Shakunthala et al. (2006). In the present study, PCR was performed with listeriolysin primer for the detection of *L. monocytogenes* in pure culture and artificially contaminated milk. Detection limit of PCR in pure culture broth of *L. monocytogenes* was $10^4$ CFU/ml (**Figure 28**) and in artificially spiked in milk the detection limit was $10^5$ CFU/ml (**Figure 29).**

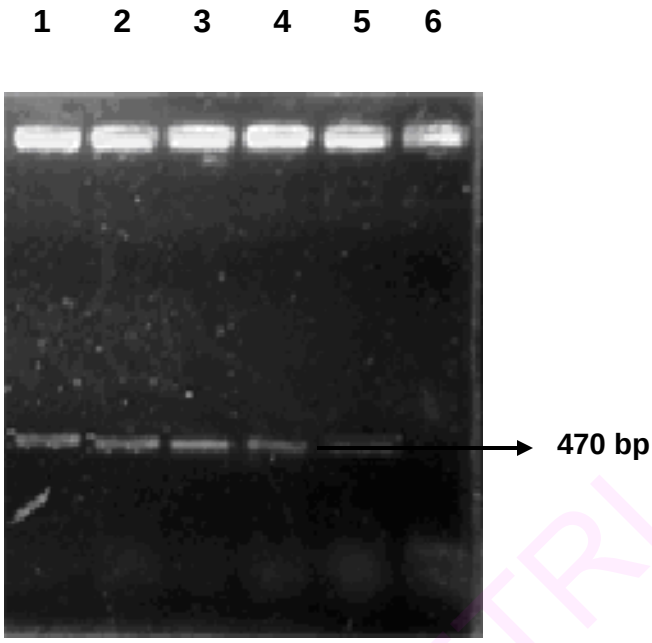


**Figure 28** Agarose gel electrophoretic pattern of PCR products showing amplicons with listeriolysin primers in respect of individual cell populations of *L*. *monocytogenes* CFR 1338 taken in saline; Lanes **1**, $10^8$ CFU/ml; **2**, $10^7$ CFU/ml; **3**, $10^6$ CFU/ml; **4**, $10^5$ CFU/ml; **5**, $10^4$ CFU/ml; 6, $10^3$ CFU/ml

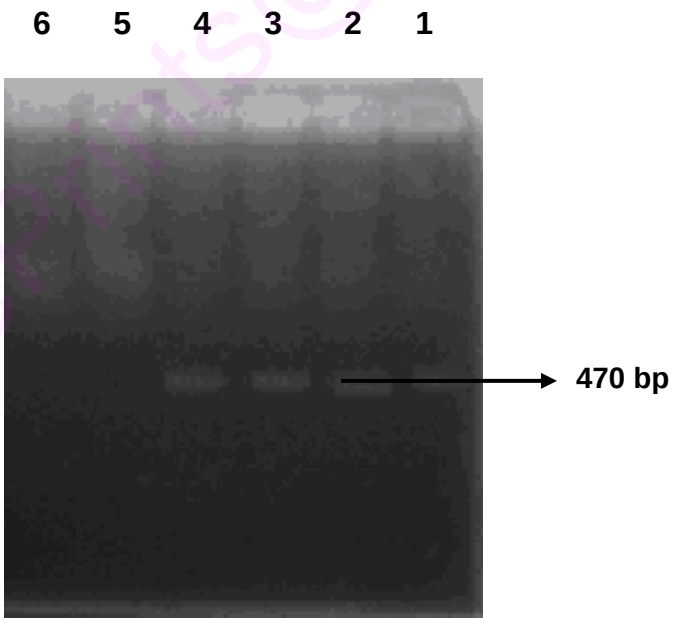


**Figure 29** Agarose gel electrophoretic pattern of PCR products showing amplicons with listeriolysin primers in respect of individual cell populations of *L*. *monocytogenes* CFR 1338 taken in skim milk; Lanes **1**, $10^8$ CFU/ml; **2**, $10^7$ CFU/ml; **3**, $10^6$ CFU/ml; **4**, $10^5$ CFU/ml; **5**, $10^4$ CFU/ml; **6**, $10^3$ CFU/ml

Competitive PCR was carried out by Choi and Hong (2003) using *hly*A gene and detected $10^3$ CFU/0.5 ml of milk. Rodriguez –Lazaro et al. (2004) has used *hly*A in real time PCR for the detection of pure *L. monocytogenes*. FRET-PCR was carried out by Koo and Jaykus (2003) using *hly* gene and detected 500 CFU/ml of pure cultures of *L. monocytogens*. Klein and Juneja (1997) had used *hly*A gene in RT PCR for the detection of *L. monocytogenes* to the level of 10-15 CFU/ml.

A multiplex PCR assay was carried out to detect all the *Listeria spp* by Bubert et al. (1999) using the iap gene encoding the protein p60. They have used the common reverse primer Lis 1B and different forward primers for the identification of individual *Listeria* spp. in pure culture .A multiplex PCR method was developed for simultaneous detection of *Salmonella* spp., *L. monocytogenes,* and *Escherichia coli O157:H7* in meat samples by Kawasaki et al. (2005). In this study, Multiplex PCR was carried out using the primers for haemolysin and listeriolysin to identify pure *L. monocytogenes CFR 1338* to the limit of $10^6$ CFU (**Figure 30**).

Nested PCR was carried out by Herman (1995) using *hly*A gene for the detection of *L. monocytogenes* in artificially contaminated milk by chemical extraction of milk followed by two step PCR for the sensitive detection f 10-15 CFU/ml. In this experimental trial, the nested PCR for the detection of *L. monocytogenes* in artificially contaminated milk was achieved. The first step of reaction was carried out by haemolysin primer and the second step was by listeriolysin primers (**Figure 31**).

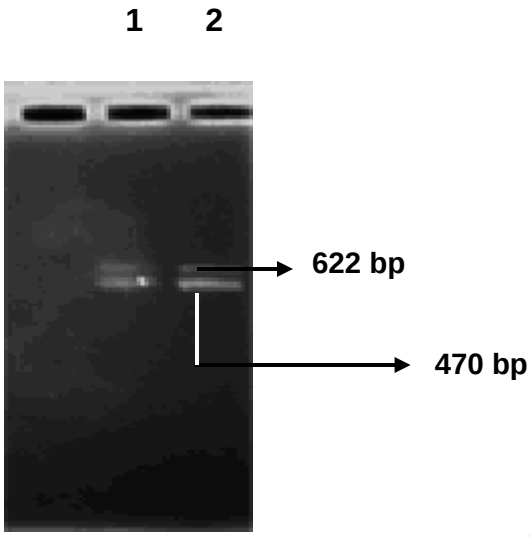


**Figure 30** Agarose gel electrophoretic pattern of PCR products showing amplicons with haemolysin and listeriolysin primers of *L*. *monocytogenes* CFR 1338 in nested PCR
Lanes **1 & 2**, Positive amplification

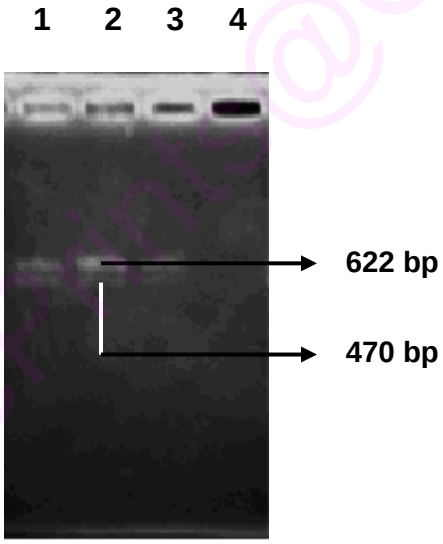


**Figure 31** Agarose gel electrophoretic pattern of PCR products showing amplicons with haemolysin and listeriolysin primers in respect of individual cell populations of *L*. *monocytogenes* CFR 1338 in multiplex PCR
Lanes **1**, $10^8$ CFU/ml; **2**, $10^7$ CFU/ml; **3**, $10^6$ CFU/ml; **4**, $10^5$ CFU/ml

A rapid and sensitive method for the direct detection of *L*. *monocytogenes* from milk has been developed by Amagnliani et al. (**year?**) based on a magnetic capture hybridization procedure for selective DNA purification, followed by PCR identification using *hly*A gene. Immunomagnetic seperation, followed by PCR was used by Bilir Ormanci et al. (2008) for the detection of *L. monocytogenes* in turkey meat. In this study, experimental trials were undertaken to optimize immuno PCR using the Sepharose beads coated with IgG and IgY for the capture of *L. monocytogenes* CFR 1338 in artificially spiked skim milk. PCR was performed using the listeriolysin primera (**Figure 32**).

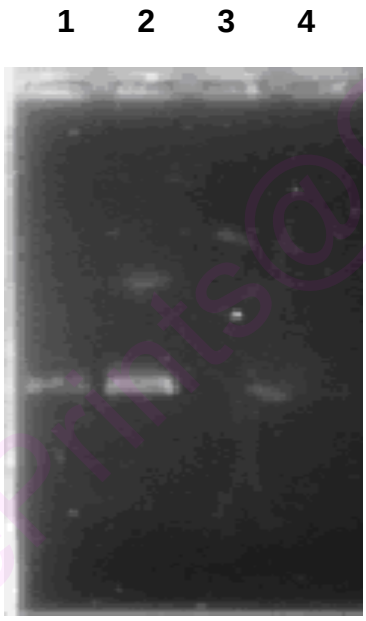


**Figure 32** Agarose gel electrophoretic pattern of PCR products showing amplicons with listeriolysin primers in respect of individual cell populations of *L*. *monocytogenes* CFR 1338 using IgG coated immunobeads and PCR performed without any prior enrichment in culture broth

Lanes **1**, $10^8$ CFU/ml; **2**, $10^7$ CFU/ml; **3**, $10^6$ CFU/ml; **4**, $10^5$ CFU/ml

In the background of aspects relating to the detection of potential toxigenic / pathogenic cultures of *L*. *monocytogenes* and other pathogenic species like *L*. *ivanovii* and *L*. *seeligeri*, the PCR methods optimized in this study in respect of primers designed and applied in two systems provide ample opportunities to bring forth the combination of immunological and molecular methods in one capsule for the food safety programmes.

## 4.4 ASSESSING THE VIRULENCE TRAITS IN *LISTERIA* SPECIES

*Listeria* shares distinguishing characteristics with closely related strains which are non-pathogenic to humans and thus, it is often difficult to clearly establish the identity of presumptive isolates. Being an opportunistic bacterial pathogen, *L. monocytogenes* demonstrates significant strain variations in virulence and pathogenicity. *Listeria* spp. have been documented to infect at least 54 species of mammalian, vertebrate and invertebrate hosts and are found to survive and persist under diversified natural habitats. The pathogenic potential of a given *L. monocytogenes* strain has been determined by mouse virulence assay, wherein use of large number of mammals is difficult for logistical, ethical and financial reasons. Recently, several non mammalian models of infection like *Danio rerio*, *Arabidopsis thaliana*, *Dictyostelium discoideum* and *Caenorabditis elegans* are being developed as alternate models to assess pathogenicity.

The use of *Drosophila* as a model offers several unique advantages as it has been studied and used in genetic analysis for almost nearly a century. Several studies have established striking similarities in respect of innate immune response between the fruit flies and mammals. *Drosophila melanogaster* has been previously developed as a model for *Pseudomonas aeurginosa*, *Mycobacterium marinum*, *Staphylococcus aureus* and *L*. *monocytogenes*. In this background, *Drosophila* has been used as a model of infection to assess the virulence trait present among the native isolates of *Listeria* spp.

### 4.4.1 MATERIALS

All the glasswares used in the present investigation were either sterilized by autoclaving at 121°C for 20 min or by dry sterilization at 180°C for 4 h in a hot-air oven. Gamma-irradiated sterile disposable petri plates, and dehydrated culture media procured

from HiMedia laboratories, Mumbai, India. The media were prepared according to the manufacturer's instructions and sterilized by autoclaving at 121°C for 15 min. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

The specific requisites in respect of specific fine biochemicals, reagents, related buffers and solvents used in this experimental study were obtained from Sigma-Aldrich, Bangalore, India; Bangalore Genei, Bangalore, India; Sisco Research Laboratories, Mumbai, India; and Qualigens, Mumbai, India.

#### 4.4.1.1 Culture bottles, needles and other materials

These included 30 quarter pint culture bottles, 27 Gauze needles and glass capillaries blown to thickness less than 31 Gauze. Gauze needles and glass capillaries were obtained from NCBI, Bangalore, India. Culture bottles were cleaned well, dried and autoclaved for use in experiments. Similarly, needles and capillaries were also sterilized prior to use the experimental trials.

#### 4.4.1.2 Culture medium for *Drosophila*

This culture medium was known as Çream of wheat medium and the composition was as follows:

| Ingredients | Weight in g/ml |
|---|---|
| Soji | 100 |
| Jaggery | 100 |
| Agar agar | 10 |
| Propionic acid | 7.5 |
| Distilled water | 1000 |
| Yeast granules | 0.5 |

Jaggery in 100 g was dissolved in 1000 ml of Milli-Q water and boiled. Ten grams of agar was mixed with 100 g of soji and added slowly into the boiling mixture and cooked for 5 min. The media was cooled to 60°C and 7.5 ml of propionic acid was added and poured into pre-strerilized 30 quarter pint culture bottles.

#### 4.4.1.3 *Drosophila* cultures and its maintenance

*Drosophila melanogaster* was obtained through courtesy from the National *Drosophila* collection centre, Manasagangothri, University of Mysore, Mysore. Wild type *D. melanogaster* flies were maintained on standard fly culture media at 25°C. Flies were maintained in 30 quarter pint culture bottles with yeast granules added at the time of fly transfer. Flies were sub-cultured to fresh medium once in every 15 d.

#### 4.4.1.4 Bacteriological media

*4.4.1.4.1 Brain heart infusion (BHI) broth and agar*

This was prepared as described under **4.1.1.5.2** (**A**) and used in this study.

*4.4.1.4.2 Listeria Oxford Agar*

This was prepared as described under **4.1.1.5.6** and used in this study.

#### 4.4.1.5 Bacterial cultures and inoculum preparation

The following were the bacterial cultures used in this experimental study:

**Table 14** Test cultures used in this experimental study

| Culture | Source |
|---|---|
| *Listeria monocytogenes* Scott A, *L. monocytogenes* V 7 and *L*. *innocua* Prd. 01 | Dr. Arun K. Bhunia, Purdue University, United States of America |
| *Listeria ivanovii* Mng 01, *L*. *seeligeri* Mng 02, *L*. *grayi* subsp. *grayi* Mng 03 and *L*. *grayi* subsp. *murrayi* Mng 04 | Dr. I. Karunasagar, College of Fisheries, KVAFS University, Mangalore, India |

| | |
|---|---|
| *Escherichia coli* MTCC 118 and *Yersinia enterocolitica* MTCC 859 | Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India |
| *Bacillus cereus* F 4810 | Dr. J.H. Kramer, Public Health Laboratory, Colindale, United Kingdom |
| *Staphylococcus aureus* FRI 722 | Dr. S. Notermans, Public Health Laboratory, The Netherlands |
| *L*. *monocytogenes* CFR 1338; *L*. *ivanovii* CFR 1344 and *L*. *grayi* subsp. *grayi* CFR 1352 | Native isolate from milk powder, raw meat and raw vegetable, respectively (from this study - **Tables of 4.1**) |

The cultures were maintained at 4°C on brain heart infusion (BHI) agar slants and subcultured at 15 d intervals. The cultures were propagated twice in BHI broth at 37°C prior to use in experiment.

Prior to use in experiments, the individual cultures of *Listeria* spp. as listed in **Table 14** were successively propagated twice in BHI broth for 24 h at 37°C in an orbital shaker incubator (Alpha Scientific Co., Bangalore, India) at 140 rpm. Cell suspensions of these cultures were individually prepared from 20 h old BHI culture broth (grown under conditions previously described) by centrifugation at 8000 rpm for 20 min at 4°C (Superspin R-V/$F_M$, Plasto Crafts, Mumbai, India). The harvested cells were resuspended in sterile 10 ml aliquots of 0.85% saline and stored in sterile screw-capped tubes at 4°C until further use.

#### 4.4.1.6 Diluent

Requisite quantities of normal saline of 0.85% were dispensed in suitable glass containers and autoclaved.

### 4.4.2 METHODOLOGY

***All experimental trials were carried out independently, in triplicates / duplicates, as the case may be and mean values with requisite statistical analysis were applied in presenting the results.***

#### 4.4.2.1 Method of infection to flies

Anaesthetization/etherisation

Prior to undertaking the infection process, drosophila flies were anaesthetized with little dose of ether. Flies were transferred to etherizing bottle (wide mouthed cork fitted bottle with thin cotton pad) and anaesthetized for 60 s by gentle shaking.

Infection

For infection, adult flies of 3-6 days were used. Flies were infected either in thorax region or abdominal region. Two different types of needles 27 gauze needles (Mansfield et al. 2003) and ultra thin needles by NCBI Bangalore were used for infection. Needles dipped in bacterial solution were used to prick the flies. Needles dipped in bacterial growth media or saline were used for mock infection. Death of the flies was used as the indication of infection. The presence of *L. monocytogenes* in the dead flies was assessed by plating the crushed dead flies after appropriate dilutions.

#### 4.4.2.2 Determination of infective dose of *L. monocytogenes* CFR 1338

The native isolate of *L. monocytogenes* CFR 1338 was used in this study, as a means to assess the potential of native food isolates to harbour toxigenic / pathogenic traits in them. The culture was grown for 24 h in 10 ml BHI broth and pelleted to get the pure culture. The pellet was serially diluted in 0.85% saline to get different cell numbers. The flies were infected in the abdominal region with approximately 0.2-0.5 ul of different dilutions. Ten flies each were taken for individual cell numbers. The flies were washed to

remove external bacteria and crushed with a glass rod by adding liquid nitrogen. The crushed flies were dissolved in 0.1 ml saline and surface plated on *Listeria* oxford agar medium. The number of *Listeria* colonies appearing in the incubated plates was counted to assess the infective dose. Flies infected with saline were taken as control. The average count was used to find the intake of *L. monocytogenes*.

#### 4.4.2.3 Determination of growth stage of *L. monocytogenes* for infection

The culture of *L. monocytogenes* CFR 1338 was grown on BHI broth. The cells were pelleted at log and stationary phases of growth. The initial dosage of each growth stage was determined by crushing the flies immediately after infection and washing. The infected flies were allowed to grow after infection till death.

#### 4.4.2.4 Determination of time course of infection of *D. melanogaster*

The BHI broth culture of *L. monocytogenes* CFR 1338 in log phase was introduced into flies by pricking in abdomen region. The cell numbers of *L. monocytogenes* introduced was determined by grinding the flies immediately after the infection and subjecting to viable populations of this organism by plating in a selective medium. The growth of *L. monocytogenes* in the flies was determined by plating the flies every day from first day to tenth day.

#### 4.4.2.5 Pathogenicity of *Listeria* spp. and other bacteria on *Drosophila*

*Different Listeria* spp. including reference cultures and also native food isolates like *L. monoctogenes scott A*, *L. monocytogenes* CFR 1338, *L. innocua*, *L. seeligeri*, *L. ivanovii*, *L. grayi* subsp. grayi, *L. grayi* subsp. *murrayi* and other Gram positive and negative bacteria like *B. cereus* F 4810, *S. aureus* FRI 722, *E. coli* MTCC 118, *Y.*

*enterocolitica* MTCC 859 were used to determine minimum infective dose to infect the flies.

### 4.4.3 RESULTS AND DISCUSSION

The major objective of this study was to assess the presence of virulence and/or toxigenic traits among the native isolates of *Listeria* spp., which prevail in a wide range of foods at very low viable populations in *Drosophila* model. Similar experiments were carried out earlier by Mansfield et al. (2003) to demonstrate *Drosophila* as a potential model for *L*. *monocytogenes* host pathogen interactions. The ability of *L*. *monocytogenes* to cause lethal infection in fruit flies with extensive bacterial replication inside the fly was established

In the present study, experiments were performed to determine the infecting region of *D. melanogaster*. The *D. melanogaster* populations were pricked with two types of needles in thoracic and abdominal regions with BHI broth and saline. The survival of 64% flies against 53% of flies, irrespective of the infecting region, showed the fine glass needles from NCBI were ideal for the infection. The survival rate of flies was more when infected in abdominal region compared to thoracic region. Nearly, 94% flies survived when pricked in the abdominal region as against to that of 34% flies infected in the thoracic region when infected with glass needles (**Table 15**). A similar type of infection of *D. melanogaster* in thoracic region was documented by Manfield et.al. (2003).

The infective dose of *L. monocytogenes* CFR 1338 for *D. melanogaster* was determined by infecting and plating the different cell populations of *L. monocytogenes*. The value was calculated from the determination of CFU per fly immediately after

infection with $10^8$ CFU/ml cultures. The infective dose was found to be 0.2-0.6 nanolitres. The growth stage of bacteria did not show much difference in infection when the experiments were carried out with log and stationary phases of the culture (**Figure 33**).

**Table 15** Mock infection of *D. melanogaster* with BHI broth in different body regions with needles of assorted sizes

| | | No. of flies infected | No. of flies survived | % survival |
|---|---|---|---|---|
| 31 gauze needle | | | | |
| | abdomen | 25 | 20 | 80 |
| | | 25 | 18 | 72 |
| | thorax | 25 | 7 | 28 |
| | | 25 | 8 | 32 |
| Glass needle (from NCBI) | abdomen | 25 | 23 | 92 |
| | | 25 | 24 | 96 |
| | thorax | 25 | 9 | 36 |
| | | 25 | 8 | 32 |

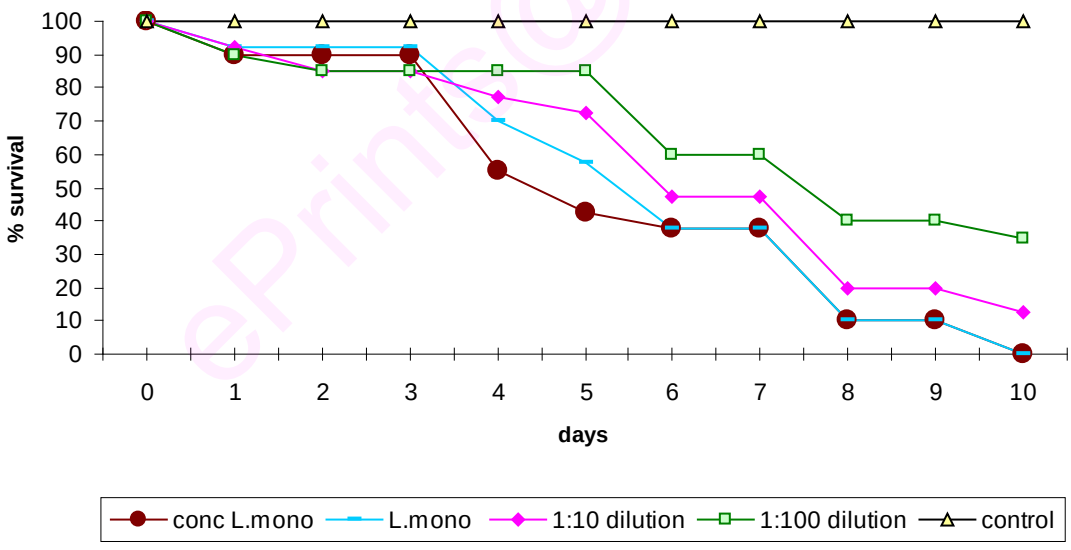


**Figure 33** Determination of *L. monocytogenes* infection lethality on *D. melanogaster*
Time course of infection of adult *D. melanogaster* with *L. monocytogenes* CFR 1338 till death of the flies by infection. The values plotted are the average of four replicate experiments, each with 10 flies

The death of flies was found to be progessive. The death pattern started from third day. All flies were killed in 8-10 d of post-infection with *L. monocytogenes* CFR 1338. When $10^8$ CFU/ml cells were used the flies died in 8-10 d. As the cell populations of test organism decreased, there was also a decrease in the death rate of flies. Infection of flies with 24 h old grown culture showed 90% death on eighth day and 100% on tenth day (**Figure 34**).

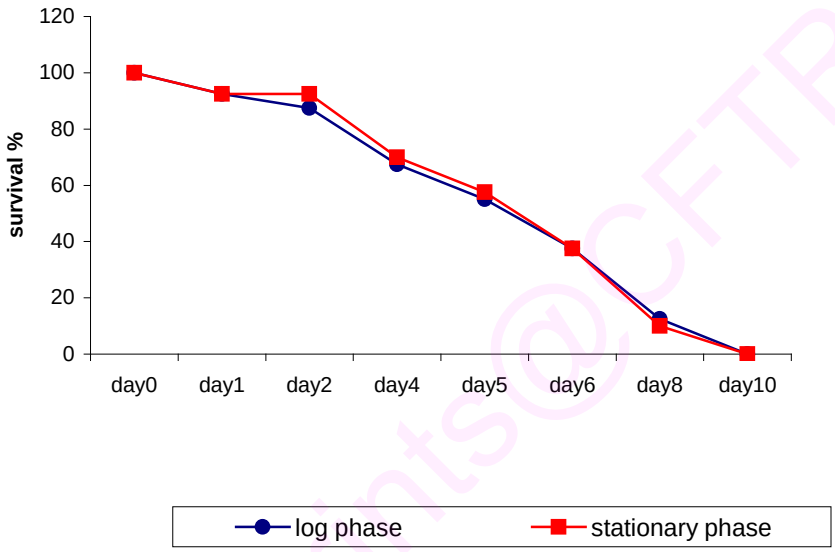


**Figure 34** infection of *D. melanogaster* with different growth stages of *L. monocytogenes* CFR 1338. The values plotted are the average of four replicate experiments, each with 10 flies

*Listeria monocytogenes* CFR 1338 was found to grow exponentially until the time of fly death, with number of viable bacteria reaching as high as $10^6$ CFU per fly. Homogenates of individual flies were prepared at various time intervals after infection and before death (**Figure 35**). The culture of *L. monocytogenes* was capable of rapid multiplication within infected *D. melanogaster*, indicating that this organism was able to

exploit host physiology and access the nutrients. The doubling time of *L*. *monocytogenes* can be partially compared to its doubling time in nutrient rich broth cultures, a phenomenon similar to that observed for bacterial growth within mammalian cells. (Mansfield et al. 2003).

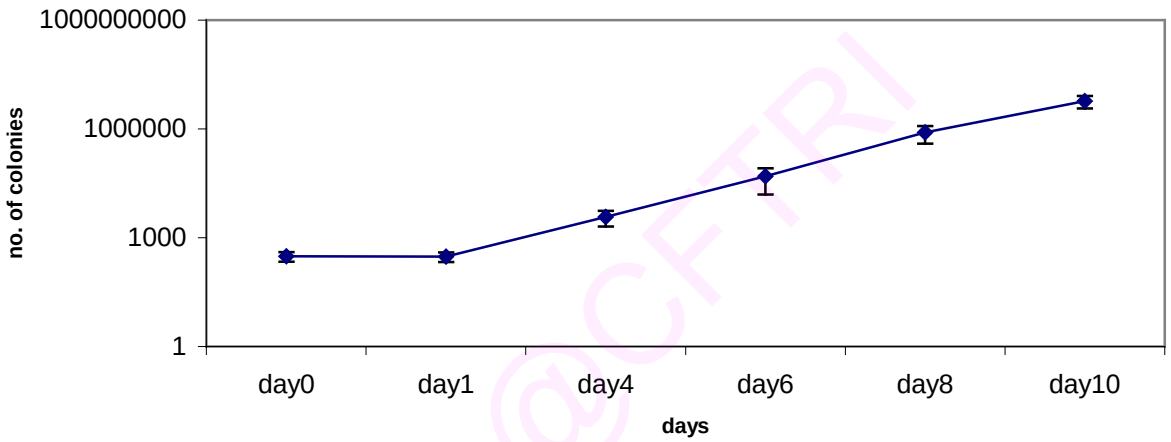


**Figure 35** Growth of *L. monocytogenes* CFR 1338 in adult *D. melanogaster*
Homogenates of individual flies were made at various time points after infection before death. Each time point represents the average bacterial CFU per fly including standard error obtained from 10 flies from 4 individual experiments

Only pure cultures or the cultures grown in minimal media was effective in *Drosophila* infection. With the culture of *L. monocytogenes* CFR 1338 grown in BHI broth, more than 60% flies survived upto 5 d. When *L. monocytogenes* spiked in milk was used for infection, death started from the very first day and only 35% flies survived by 5$^{th}$ day, but all the flies died on 10$^{th}$ day (**Figure 36**).

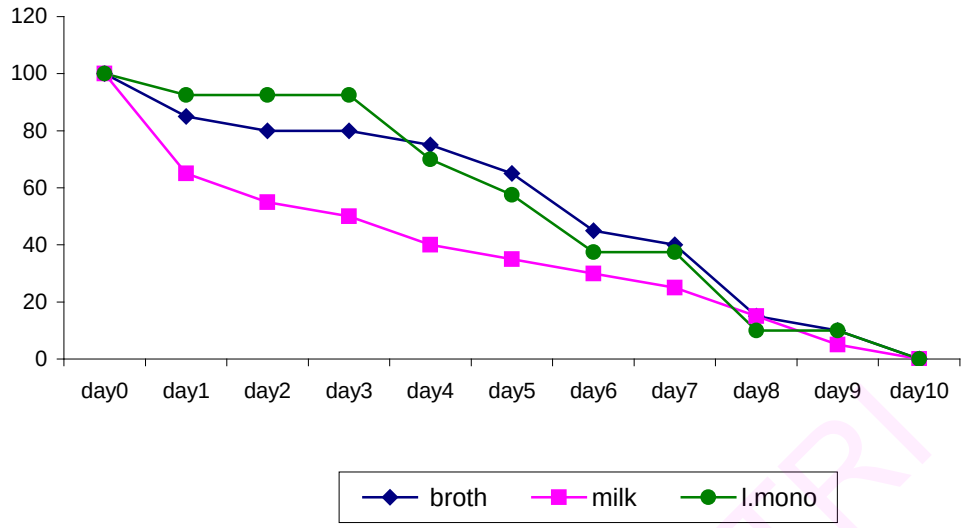


**Figure 36** Infection of *D. melanogaster* with artificially inoculated *L. monocytogenes* CFR 1338; Values plotted are the average of four replicate experiments, each with 10 flies

Pathogenicity of *L. monocytogenes* and *L. ivanovii* towards *D. melanogaster* is shown in **Table16 and 17**. The culture of *L. monocytogenes* CFR 1338 was found to be 100% pathogenic, while *L. ivanovii* showed only 50% pathogenicity at 10$^{th}$ day. Pathogenicity of other Gram positive and Gram negative bacteria used were not well established. Even though *S. aureus* infection to *D. melanogaster* was established by Needham et al. (2004), it was found that *S. aureus* killed the flies in 15-20 days of infection. *Psuedomonas aeroginosa* was found to kill *D*. *melanogaster* in less than 50 h of infection. (D' Argeno et al. 2001), *Mycobacterium marinum* killed *D. melanogaster* in 8-9 days of post infection (Dionne et al. 2003). Non-human pathogenic organisms like *E. coli* and *M. luteus* were found to replicate freely within fly circulation without killing the fly. (Hoffmann and Reichhart, 2002).

**Table.16** Pathogenicity of different reference bacterial cultures towards *Drosophila*

| Bacteria | Pathogenicity towards *Drosophila* | Time course of infection | Mortality |
|---|---|---|---|
| L. monocytogenes ScottA | + | 8-10 days | 100% |
| L. monocytogenes V7 | + | 8-10 days | 100% |
| *Listeria* innocua Prd. 01 | - | | |
| *Listeria* seeligeri Mng 02 | - | | |
| *Listeria* ivanovii Mng 01 | ± | 8-10 days | 50% |
| *Listeria* grayi Mng 03 | - | | |
| *Listeria* murrayi Mng 04 | - | | |
| *Escherchia coli* MTCC 118 | - | | |
| *Bacillus cereus* F4810 | - | | |
| *Staphylococcus aureus* FRI 722 | ± | 15-17 days | |
| *Yersinia enterocolitica* MTCC 859 | - | | |

**Table 17** Infection of *D. melanogaster* with native isolates of *Listeria* spp.

| Native isolates | Pathogenicity towards *Drosophila* | Time course of infection | Mortality |
|---|---|---|---|
| *L. monocytogenes* CFR 1338 | + | 8-10 days | 100% |
| *L. ivanovii* CFR 1344 | ± | 8-10 days | 50% |
| *Listeria* spp. | | | |
| *Listeria* spp. | | | |
| *Listeria* spp. | | | |
| *L. ivanovii* | ± | 8-10 days | 50% |
| *L. monocytogenes* | + | 8-10days | 100% |
| *L. grayi* CFR 1352 | - | | |
| *L. ivanovii* | ± | 8-10 days | 50% |

From the above experimental data, it was observed, that *Drosophila* can be used as an efficient model for the determination of pathogenicity of *L. monocytogenes*. The atypical *Listeria* strains with variable biochemical characters were examined for virulence, as few uncommon *L. monocytogenes* in deli turkey caused listeriosis during 2002 in United Stated (CDC 2002). The difference in pathogenic potential may be due to the ability of the pathogen to infect, spread from cell to cell and intracellular replication. Haemolysis may be the basic criteria for pathogenecity of bacteria towards *Drosophila*.

## 5.0 SUMMARY AND CONCLUSION

In the background of emerging issues of food safety programmes at the national scenario as well as to meet stringent requirements of microbial safety of foods at the global platform, the present study undertook attempts to assess the prevalence of *Listeria* spp. in a diverse range of foods available locally and evaluate the potentiality of these food isolates to harbour any toxigenic / virulence traits as determined by optimised immunoassays and molecular methods.

Prevalence of *Listeria* species in raw milk, milk-based products, raw meat, meat-based products, raw vegetables, and cereal-legume-based products was assessed by non-selective cold enrichment technique, followed by selective enrichment and plating on selective agar. The presumptive positive *Listeria* isolates were confirmed to their genus and species by conventional methods of morphological, cultural and biochemical characteristics. On the basis of preliminary characterization, the study showed listerial incidence to be of 26% in raw milk and 20% in raw meat. Almost all the isolates obtained from raw milk were identified to be *L. grayi* subsp. grayi.

At the same time, samples of milk-based food commodities revealed the presence of potent pathogenic species of *Listeria*. Although only one or two isolates among several of the presumptive *Listeria* isolates were confirmed as pathogenic / virulent cultures, the presentation in terms of percent incidence looks higher in the background of number of samples analysed under each

category. The major ones were those of *L*. *monocytogenes* to an extent of 37% in milk-based products and 11% in raw vegetables, respectively. In the case of raw meat samples, the prevalence of *L*. *ivanovii* was to an extent of 25%. The samples of cereal/legume-based foods and processed meat-based foods were devoid of any *Listeria* spp. On an overall situation, the data obtained gave a probable projection of low numbers of *L*. *monocytogenes* and other related species of *Listeria* to prevail in food products. This goes to support the fact that outbreaks of listeriosis is very much uncommon in the Indian scenario, except for one or two reports about presence and/or absence of *Listeria* spp. in foods.

Among the native food isolate of *Listeria* spp. obtained in this study, cultures of *L*. *monocytogenes* CFR 1338, *L*. *ivanovii* CFR 1344 and *L*. *grayi* subsp. *grayi* CFR 1352 were selected to evaluate for their toxigenic / virulent potential.

Listeriolysin O (LLO), a sentinel molecule for the detection of both disease caused and protection elicited by pathogenic *Listeria* was purified to homogeneity through solvent precipitation of protein from the culture filtrate, followed by fractionation through Biogel P-100 and Sephadex G-75 column chromatography. Prior to partial purification and characterization, the potent pathogenic isolates of *Listeria* spp. were subjected to haemolytic assay with RBCs of human and rabbit. The haemolytic activity was observed to be highest at 36 h of incubation period. One of the native food isolate of *L*. *monocytogenes* CFR 1338 showed

appreciable degree of haemolysis. The purified active fraction of LLO from *L*. *monocytogenes* Scott A had a pH optimum of 5.5 and incubation temperature of 37°C. In SDS-PAGE, the purified fraction appeared to have a molecular mass of 58 kDa. The use of such a purified active fraction enabled in raising antibody specific to LLO of *L*. *monocytogenes*, both in rabbits (IgG) and poultry (IgY), following the established immunization protocols. The antibody (IgY) produced in poultry, which was through egg yolk had a higher specificity. All the requisite parameters to develop immunoassays based on IgG and IgY were optimised and applied in indirect ELISA, Dot Blot and Colony Blot for the detection of a native food isolate, *L*. *monocytogenes* CFR 1338, both in culture broth and skim milk.

In the case of varying cell populations of this native isolate of *L*. *monocytogenes* CFR 1338 taken in saline, it was possible to detect a minimum cell population of $10^4$ CFU/ml in ELISA with IgG and IgY. Similarly, cell populations spiked into skim milk resulted in the detection limit of $10^5$ CF/ml using the same protocol of ELISA. Besides, the same findings were also confirmed in Dot Blot assay. Further, the antibodies, particularly those obtained in poultry were utilized to develop immunobeads for application in the recovery of specific and targeted isolates of *L*. *monocytogenes* present in very low numbers amongst the inherent microflora of a food product.

*Listeria monocytogenes* and *Listeria* spp. were subjected to PCR with the pathogenic locus specific primer haemolysin and species-specific primers

listeriolysin, ivanolysin and seeligerolysin. The detection of individual isolates of *Listeria* spp by the use of specific primers has shown the specificity of the designed primers. Detection of *L*. *monocytogenes* to the level of $10^3$ CFU has revealed the rapidity and sensitivity of the PCR method. Detection of pathogenic isolate of *L. monocytogenes* CFR 1338 for multiple primers like that of haemolysin and listeriolysin was established in multiplex and nested PCRs. The immunobeads, which enabled to separate cells of *L*. *monocytogenes* was effective in its application to detect specific and targeted species even from a food matrix such as skim milk. The single colony PCR performed without isolation of template DNA was very rapid and can be used as an alternative to conventional characterization of the organism subsequent to isolation.

The virulence of the native isolates of *Listeria* spp. along with reference cultures of *Listeria* spp. was established in *Drosophila*. The findings of present study revealed, that *Drosophila* could be used as a model to assess for virulence determination in *Listeria* spp. The death of the flies within 10 days of infection has shown that isolates of *Listeria* present in food and other related habitats are potent risk causing species. The positive findings of these experimental trials give ample opportunities to use *Drosophila* as an alternate to other animal models.

The findings of present approach towards assessing the prevalence of a major foodborne pathogenic bacterial species like *L*. *monocytogenes* in foods

being commonly consumed by human populations in this part of sub-continent may not look very alarming. However, the methods used and protocols followed could result in 2-3 potent pathogenic/virulence trait-harbouring isolates provide enough food for food technologists, public health microbiologists and food regulators to find workable solutions towards obtaining a safe and healthy food not only devoid of *L*. *monocytogenes* and also other pathogenic bacterial species.

## 6.0 BIBLIOGRAPHY

Allerberger, F. **(2003).** Listeria: growth, phenotypic differentiation and molecular microbiology. FEMS Immunol. Med. Microbiol. **35**, 183–189.

Amalgiani,G., Omiccioli,E., Campo,A., Bruce,I.J., Brani,G. and Magnani,M. (**2006**). I.Appl.Microbiol. **100**, 375-383

AOAC International (1995) Official Method of Analsis, 16th edn

Arimi, S.M., Ryser, E.T. and Donnelly, C.W. **(1997)**. Dairy cattle and silage as potential sources of *Listeria* ribotypes common to dairy processing facilities. J. Food Prot. **60**, 811

Armstrong, B.A. and Sword, C.P. **(1966)**. Electron microscopy of *Listeria monocytogenes* – infected mouse spleen. J. Bacteriol. **91**, 1346-1355

Aznar, R. and Alarcon, B. **(2002)**. On the specificity of PCR detection of *Listeria monocytogenes* in food.: a comparison of published primers. Syst. Appl. Microbiol. **25**, 109-119.

Backman, A., Lantz, P., Radstrom, P. and Olcen, P. **(1999)**. Evaluation of extended diagnostic PCR assay for the detection and verification of the common causes of meningitis in CSF and other biological samples. Mol. Cell. Probes **13,** 49-60.

Baetz, A.L. and Wesley, I.V. **(1995)**. Detection of anti-listeriolysin O in dairy cattle experimentally infected with *Listeria monocytogenes*. Vet. Diagn. Invest. **7**, 82-86.

Bansal, N.S., McDonell, F.H., Smith, A., Arnold, G. and Ibrahim, G.F. **(1996)**. Multiplex PCR assay for the routine detection of *Listeria* in Food. Int. J. Food Microbiol. **33,** 293-300.

Barbuddhe, S.B., Chaudhari, S.P. and Malik, S.V.S. **(2002)**. The occurrence of Pathogenic *Listeria monocytogenes* and antibodies against Listeriolysin O in buffaloes. J. Vet. Med. **49**, 181-184.

Barbuddhe, S.B., Malik, S.V.S., Choudhary, S.P. and Gupta, L.K. **(1998)**. Kinetics of interferon gamma production and its comparison with anti - listeriolysin O detection in experimental bovine listeriosis. Vet. Res. Commun. **22**, 505-516.

Berche, P., Reich, K.A., Bonnicon, M., Beretty, J.L., Geoffery, C., raveneau, J., Cossart, R., Gaillard, L., Geslin, P., kreis, H. and Veron, M. **(1990).** Detection of anti-listeriolysin O for the serodiagnosis of human listeriosis. Lancet. **335**, 624-627.

Bergays manual of Systematic bacteriology vo2, 9th edition, 4th edition

Beumer, R.R. and Hazeleger, W.C. **(2003)**. *Listeria monocytogenes*: diagnostic problems. FEMS Immunol. Med. Microbiol. **35**, 191-197.

Beumer, R.R., Brinkman, E., **(1989)**. Detection of *Listeria* spp. with a monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA). Food Microbiol. **6**, 171– 177.

Bhadki, S., Tranum-Jensen, J. and Sziegoleit, A. **(1985)**. Mechanism of membrane damage by streptolysin O. Infect. Immun. **47**, 52-60.

Bhagwat, A.A. **(2003)**. Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real time PCR. Int. J. Food Microbiol. **84**, 217-224.

Bhunia, A.K. **(1997)**, Antibodies to *Listeria monocytogenes*. Critical rev. microbiol. **23**, 77-107.

Bhunia, A.K., Ball, P.H. and Johnson, M.G. **(1992)**. A 20-24 H Microcolony-immunoblot technique to directly detect and enumerate *Listeria monocytogenes* inoculated into foods. J. Rapid Methods and Automation microbiol. **1**, 67-82.

Bhunia, A.K., Ball, P.H., Faud, A.T., Kurz, B.W., Emerson, J.W. and Johnson,M.G. **(1991)**, Development and characterization of a monoclonal antibody specific for *Listeria monocytogenes* and *Listeria innocua*. Infect. immun. **59**, 3176

Bickley, J., Short,J.K. Mc Dowell,D.G. and Parkes,H.C. (1996).Polymerase chain reaction(PCR) detection of Listeria monocytogenes in diluted milk and reversal of PCR inhibition caused by calcium ions. Ltt.Appl. Microbiol.22,153-158

Bilir ormanci,F.S., Erol,I., Ayaz,N.D., Iseri O. and Sariguzel,D. **(2008).** Immunomagnetic seperation and PCR detection of Listeria monocytogenes in turkey meat and antibiotic resistance of isolates. Br Poult Sci **49(5)**, 560-565

Blais, B.W. and Phillippe, L.M. **(1993)**. A simple RNA probe system for analysis of *Listeria monocytogenes* polymerase chain reaction products. Appl. Environ. Microbiol. **59**, 2795-2800.

Blais, B.W., Turner, G., Sooknanan, R. and Malek, L.T. **(1997)**. A nucleic acid sequence based amplification system for detection of *Listeria monocytogenes* hlyA sequences. Appl. Environ. Microbiol. **63**, 310-313.

Bohnert, M., Dilasser, F., Dalet, C., Mengad, J. and Cossart, P. **(1992)**. Use of oligonucleotides for the direct enumeration of *Listeria monocytogenes* in food samples by colony hybridization and rapid detection by PCR. Res. Microbiol. **143**, 271-280

Bonev, B.B., Gilbert, R.J., Andrew, P.W., Byron, O. and Watts, A. **(2001).** Structural analysis of the protein/lipid complexes associated with pore formation by the bacterial toxin pneumolysin. J. Biol. Chem. **276**, 2611-2616.

Boujemma-paterski, R., Gouin, E. and Hansen, G. **(2001)**. *Listeria* protein ActA mimics WASP family proteins: it activates filament barbed end branching by Arp2/3 complex. Biochemistry **40,** 11390-11404.

Braden, C.R. **(2003)**. Listeriosis Pediatr. Infect. Dis. J. **22,** 745-746.

Breitling, F. and Dubel, S. **(1999).** Recombinant antibodies. John Wiley and Sons Inc., New York, 154.

Bsat, N. and Batt, C.A. **(1993)**. A combined modified reverse dot blot and Nested PCR assay for the specific non-radioactive detection of *Listeria monocytogenes.* Mol. Cell. Probes **7**, 199-207.

Bubert, A., Kohler, S., and Goebel, W. **(1992)**. The homologous and heterologous regions within the iap gene allow genus and species-specific identification of *Listeria* spp. by polymerase chain reaction. Appl. Envoron. Microbiol. **58**, 2625-2632.

Burtscher, C. and Wertz, S. **(2003)**. Evaluation of the use of PCR and Reverse transcriptase PCR for the detection of pathogenic bacteria in biosolids from anaerobic dijestors and aerobic composters. Appl. Environ. Microbiol. **69**, 4618-4627.

Call, D.R., Borucki, M.K. and Loge, F.J. **(2003)**. Detection of bacterial pathogens in environmental samples using DNA microarrays. J. Microbiol. Methods **53**, 235-243.

Cammilli, A., Tinle, L.G. and Portnoy, D.A. **(1993)**. Dual role of plcA in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8,** 143-57.

Caroll, S.A., Carr, L.E., Mallinson, E.T., Lamichanne, C., Rice, B.E., Rollins, D.M. and Joseph,S.W. **(2000).** A colony lift immunoassay for the specific identification and quantification of *Listeria monocytogenes*. J.microbiol.methods **41**, 145-153

CDC, (1998). Preliminary food net data on the incidence of infections with pathogens transmitted commonly through food--- 10 states, United States,1998. , MMVR weekly 47, 1085-1086

CDC, (1999), Preliminary food net data on the incidence of infections with pathogens transmitted commonly through food--- 10 states, United States,1998-1999. MMVR weekly 47, 1117-1118

CDC, (2002), Preliminary food net data on the incidence of infections with pathogens transmitted commonly through food--- 10 states, United States,2002. ,MMVR weekly 51(15), 325-9

CDC, (2006), Preliminary food net data on the incidence of infections with pathogens transmitted commonly through food--- 10 states, United States,2005. MMVR weekly 55(14), 392-395

Chemburu, S., Wilkins, E. and abdel-hamid, I. **(2005)**. Detection of pathogenic bacteria in food samples using highly-dispersed carbon particles. Biosens. Bioelectron. **21**, 491-509.

Chen, W., Li, D., Palus, B., Wison, I. and Chadwick, V.S. **(2000)**. Detection of *Listeria monocytogenes* by polymerase chain reaction in intestinal mucosal biopsies from patients with inflamatory bowel disease and controls. J. Gastroenterol. Hepatol. **15**, 1145-1150.

Cheng, L.W. and Portnoy, D.A. **(2003).** Drosophila S2 cells: an alternative infection model for *Listeria monocytogenes*. Cell. Microbiol. **5**, 875-885

Choi, W.S. and Hong, C. H. **(2003)**. Rapid enumeration of *Listeria monocytogenes* in milk using compitative PCR. Int. J. Food Microbiol. **84**, 79-85.

Christie, R., Atkins, N.E. and Munch-Petersen, E. (1944). A note on a lytic phenomenon shown by group B *Streptococci*. Aust. J. Exp. Biol. Med. Sci. **22**, 197-200.

Churchill, R.L.T., Lee, H. and Hall, J.C. **(2005).** Rapid purification of recombinant listeriolysin O (LLO) from *Escherchia coli*. J. Ind. Micribiol. biotechnol. **32**, 355-363.

Churchill, R.L.T., Sheedy, C., Yau, K.Y.F. and Hall, J.C., **(2002).** Evolution of antibodies for environmental monitoring: from mice to plants. Anal. Chim. Acta **468**, 185-197.

CIDRAP (2006). Centre for drug related issues USA

Ciesielski, C.A., Hightower, A.W., Parsons, S.K. and Broome, C.V. **(1998)** Listeriosis in united states, 1980-1982. Arch intern med **148**, 1416-1419.

Cocolin, L., Manzano, M., Cantoni, C. and Comi, G. **(1997)**. A PCR-microplate capture hybridization method to detect *Listeria monocytogenes* in blood. Mol. Cell. Probes **11**, 453-455.
Cocolin, L., Rantsiou, K., Iacumin, L., Cantoni, C. and Comi, G. **(2002)**. Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. Appl. Environ. Microbiol. **68**, 6273-6282.

Coffey, A., Rombouts, F.M. and Abee, T. **(1996)**. Influence of environmental parameters on Phosphatidyl choline phospholipase C production in *Listeria monocytogenes*: a convenient method to differentiate *L. monocytogenes* from other *Listeria* species. Appl. Environ. Microbiol. **62**, 1252-1256.

Colins, M.D., Wallbanks, S., Lane, D.J., Shah, J., Nietupki, R., Smida, J., Dorsch, M. and Stackbrandt, E. **(1991)**. Phylogenetic analysis of genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA . Int. J. Syst. Bacteriol. **41**, 240-246.

Comi, G., Valenti, M., Civilini, M., Fumagalli, C., Cantoni, C. and De Bertoldi, M. **(1991).** Evaluation of an enzymatic method for fast identification of *Listeria* spp. in cheese and meat products. Zentralbl. Hyg. Umweltmed. **192**, 134–145.

Curiale, M.S., Lepper, W. and Robison, B. **(1994).** Enzymelinked immunoassay for detection of *Listeria monocytogenes* in dairy products, seafoods, and meats: collaborative study. J. AOAC Int. **77**, 1472–1489.

D' Argenio, D.A., Gallagher, L. A., Berg, C.A. and Manoil, C. **(2000)**. Drosophila as a model host for *Pseudomonas aeruginosa* infection. J. Bacteriol. **183**, 1466-1471.

D'Agostino, M., Wagner, M., Vazqez-Boland, J.A., Kuchta, T., Karpiskova, R., Hoorfar, J., Novella, S., Scortti, M., Ellison, J., Murray, A., Fernandes, I., Kuhn, M., Pazlarova, J., Heuvlink, A.

and Cook, N. **(2004)**. A validated PCR-based method to detect *Listeria monocytogenes* using raw milk as a food model – towards an international standard. J. Food Prot. **67**, 1646–1655.

Darji, A., Chakraborty, T., Neibuhr, K., Tsonis, N., Wehland, J. and Weiss, S. **(1995).** Hyperexpression of listeriolysin in the nonpathogenic species *Listeria innocua* and high yield purification. J. Bacteriol. **43**, 205-212.

De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M. and Lemaitre, B. **(2002).** The toll and Imd pathways are the major regulators of immune response in *Drosophila.* EMBO J. **21**, 2568-2579.

Dhanashree, B., Otta, S.K., Karunasagar, I., Goebel, W. and Karunasagar, I., **(2003)**. Incidence of *Listeria* spp. In clinical and food samples in Mangalore, India. Food microbiol. **20**, 447-453.

Dionne, M.S., Ghori, N. and Schneider, D.S. **(2003)**. *Drosophila melanogaster* is a genetically tractable model host for *Mycobacterium marinum*. Infect. Immun. **71**, 3540-3550.

Domann, E., Wehland, J. and Rohde, M. **(1992)**. A novel bacterial virulence gene in *Listeria monocytogenes* is required for host cell microfilament interaction with homology to the praline rich region of vinculin. EMBO J. **11**, 1981-1990.

Donnelly, C.W. **(2001)**. *Listeria monocytogenes*: a continuing challenge. Nutr. Rev. **59**, 183-194.

Doumith, M., Cazalet, C., simoes, N., Frangeul, L., Jacquet, C., Kunst, F., Martin, P., Cossart, P., Glaser, P. and Buchrieser, C. **(2004)**. Portable system for microbial sample preparation and oligonucleotide microarray analysis. Appl. Environ. Microbiol. **67**, 922-928.

Dramsi, S., Kocks, C., Forestier, C. and Cossart, P. **(1993).** Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA*. Mol. Microbiol. **9**, 931.

Erdenlig, S., Ainsworth, J. and Austin, F.W. **(1999).** Production of Monoclonal Antibodies to *Listeria monocytogenes* and their application to determine the virulence of Isolates from channel catfish. Appl. Environ. Microbiol. , 2827-2832.

Farber, J. M. and Speirs, J. I. **(1987).** Monoclonal antibodies directed against the flagellar antigens of *Listeria* species and their potential in EIA-based methods. J. Food Prof. **50**, 479.

Farber, J.M. and Peterkin, P.I. **(1991)**. *Listeria monocytogenes*, a food borne pathogen. Mic. Rev. **55**, 476-511.

Farber, J.M., Sanders, G.W. and Speirs, J.I. **(1988).** Methodology for isolation of *Listeria* from foods – a canadian perspective. J. AOAC Int. **71**, 675-678.

Feldstine, P.T., Lienau, A.H., Forgey, R.L. and Calhoon, R.D. **(1997).** Assurance polyclonal enzyme immunoassay for detection of *Listeria monocytogenes* and related *Listeria* species in selected foods: collaborative study. J. AOAC Int. **80**, 775-790.

Feng. (2001). Appendix 1 Rapid Metods for detecting food borne pathogens in US Food and Drug Administration's Bacteriological Analytical Manual (online)

Fenlon, D. R. **(1999).** *Listeria monocytogenes* in the natural environment. In *Listeria, Listeriosis and Food Safety*,. 21–37. Ed E. T. Ryser & E. H. Marth. New York: Marcel Dekker

Fenlon, D.R. **(1986)**. Rapid quantitative assessment of the distribution of *Listeria* in silage implicated in a suspected outbreak of listeriosis in calves. Vet. Rec. **118**, 240

Fluit, A. C., Torensma, R., Visser, M. J. C., Aarsman, C. J. M., Poppelier, M. J. J. G., Keller, B. H. I., Klapwijk, P. and Verhoef, J. **(1993).** Detection of *Listeria monocytogenes* in cheese with the magnetic immunopolymerase chain reaction assay. Appl. Environ. Microbiol. **59**, 1289.

Fuchs, R.S. and Surendran, P.K. **(1989)**. Incidence of *Listeria* in tropical fish and fishery products. Lett. Appl. Microbiol. **9**, 49-51.

Fuzi, M. and Pillis, I. **(1962)**. Production of opacity in the egg yolk medium by *Listeria monocytogenes*. Nature **13**, 195.

Gailard, J.L., Berche, P., Mounier, J., Richard, S., Sansonetti, P.J. **(1987)**. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the erythrocyte like cell line Caco-2. Infect. Immun. **55**, 2822-2829.

Gaillard, J.L., Berche, P., Sansonetti, P. **(1986)**.Transposan Mutagenesis as a tool to study the Role of Hrmolysin in the virulence of *Listeria monocytogenes*. Infection and Immunity. **52**, 50-55.

Garrec, N., Dilasser, F., Pourcher, A.M., Perelle, S. and Fach, P. **(2003).** Comparison of a cultural method with ListerScreen plus Rapid_L.mono or PCR-ELISA methods for the enumeration of *L. monocytogenes* in naturally contaminated sewage sludge. J. Microbiol. Methods **55**, 763–773.

Gasanov, U., Hughes, D. and Hansbro, P.M. **(2005)**. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. **29**, 851-875.

Gellin, B.G., Broome, C.V., Bibb, W.F., Weaver, R.E., Gaventa, S., Mascola, L. and listeriosis study group. **(1986).** The epidemiology of Listeriosis in United States. A. J. epidemi **133**, 392-401.

Geoffroy, C., Gaillard, J.L., Alouf, J.E. and Berche, P. **(1987)**. Purification, characterization and toxicity of sulphydril-activated hemolysin O from *Listeria monocytogenes*. Infect. immunol. **55**, 1641-1646.

Giamarini, C., Andreoni, F., Amagliani, G., Casiere ,A., Barocci, S. and Magnani, M. **(2004).** Purification and characterization of a recombinant Listeriolysin O expressed in *Escherchia coli* and possible diagnostic applications. J. Bacteriol. **109**, 13-20.

Giamarini, C., Andreoni, F., Amagliani, G., Casiere, A., Barocci, S and Magnani, M. **(2003)**. High level expression of the *Listeria monocytogenes* listeriolysin O in *Escherchia coli* and preliminary characterization of purified protein. Pro exp purification. **28**, 78-85.

Gill, D.A. **(1937)**. Ovine bacterial encephalitis (Circing disease) and the bacterial genus Listerella. Aust. Vet. J. **13**, 46.

Glomiski, I.J., Gedde, M.M., Tsang, A.W., Swanson, J.A. and Portnoy, D.A. **(2002)**. The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. J. Cell Biol. **156**, 1029-1038.

Gohmann, M., Leimeister-Wachter, M., Schiltz, E., Goebel, W. and Chakraborty, R. **(1990).** Characterization of a *Listeria monocytoenes*-specific protein capable of inducing delayed hypersensitivity in Listeria-immune mice. Mol. Microbiol*.* **4**, 1091.

Gouin, E., Mengaud, J. and Cossart, P. **(1994)**. The virulence gene cluster of *Listeria monocytogenes* is also present in *Listeria ivanovii*, an animal pathogen, and *Listeria seeligeri*, a non –pathogenic species. Infect. immune **62**, 3550-3553

Gray, k.M. and Bhunia, A.K. **(2005)**. Specific detection of cytopathogenic *Listeria monocytogenes* using two step method of immunoseperation and cytotoxicity analysis. J. microb. Methods **60**, 259-268.

Groves, R.D. and Welshimer, H.J. **(1977)**. Seperation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. J. Clin. Microbiol. **5**, 559-563.

Gugnani, H.C. **(1999)**. Some food and water borne pathogens. J. Commun. Dis. **31**, 65-72.

Gupta, V., Gautham, V., Mahta, N., Kumari, I. and joshi, R.M. **(2003)**. Listeriosis in second trimester of pregnancy: case report from India. Jpn. J. Infect. dis. **56**, 60-61.

Ha, K.S., Park, S.J., Seo, S.J., Park, J.H. and Chung, D.H. **(2002)**. Incidence and polymerase chain reaction assay of *Listeria monocytogenes* from raw milk in Geongnam Province of Korea. J. AOAC Int. **81**, 817-822.

Hall, J.C., Deschamps, R.J.A. and Kreig, K.K. **(1989).** Immunoassays for the detection of 2, 4-D and picloram in river water and urine. J. Agric. Food Chem. **37**, 981-984.

Hayes, P.S., Graves, L.S., Swaminathan, B., Ajello, GG.W., Malcolm, G.B., Weaver, R.E., Ransom, R., Deaver, K., Plikaytis, B.D., Schuachat, A., Wenger, J.D., Pinner, R.W., Broome, C.V. and Group, L.S. **(1992).** comparison of three selective enrichment methods for the isolation of Listeria monocytogenes from naturally contaminated foods. J. food prot. **55**, 952-959.

Hein, I., Klein, D., Lehner, A., Bubert, A., Brandl, E. and Wagner, M. **(2001)**. Detection and quantification of the iap gene of *Listeria monocytogenes* and *Listeria innocua* by a new real time quantitative PCR assay. Res. Microbiol. **152**, 37-46.

Herman, L.M., De BlockJ, H. and Moermans, R.J. **(1995)**. Direct detection of *Listeria monocytogenes* in 25 millilitres of raw milk by two step PCR with nested primers. Appl. Environ. Microbiol. **61**, 817-819.

Hird, D.W. **(1987)**. Review of evidence for zoonotic listeriosis. Food Protect. **50**, 429-433.

Hitchins, A.D. (2003) Detection and enumeration of *Listeria monocytogenes* in food. Bacterial Analytical Manual online.

Hoffmann, A.D. and Weidman, M. **(2001)**. Comparative evaluation of culture and BAX polymerase chain reaction-based detection methods for *Listeria* spp. and *Listeria monocytogenes* in environmental and raw fish samples. J. Food Prot. **64**,1521 – 1526.

Hoffmannn, J.A. and Reichhart, J.M. **(2002).** Drosophila innate immunity: An evolutionary perspective. Nat Immunol. **3**, 121-126.

Hough, A.J., Lake, R.J., Savill, M.G., Melton, L.D. and Fletcher, G. **(2002)**. Rapid enumeration of *Listeria monocytogenes* in artificially contaminated cabbage using real time polymerase chain reaction. J. Food Prot. **65**, 1329-1332.

Hsih, H.Y. and Tsen, H.Y. **(2001)**. Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in food samples. J. Food Protect. **64**, 1744–1750.

Hudson, J.A., Lake, R.J., Savill, M.G., Scholes, P. and McCormick, R.E. **( 2001).** Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic seperation followed by polymerase chain reaction. J. Appl. Microbiol. **90**, 614-621.

.

Huijsdens, X.W., Linskens, R.K., Taspinar, H., Meuwissen, S.G., Vandenbroucke-Grauls, C.M. and Savelkoul, P.H. **(2003)**. *Listeria monocytogenes* and inflammatory bowel disease: detection of *Listeria* species in intestinal mucosal biopsies by real-time PCR. Scand. J. Gastroenterol. **38**, 332-333.

Ingianni, A., Floris, M., Palomba, P., Madeddu, M.A., Quartuccio, M. and Pompei, R. **(2001)**. Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. Mol. Cell. Probes **15**, 275 - 280.

Ito, Y., Kawamura, I., Kohda, C., Baba, H., Kimoto, T., Watanabe, I., Nomura, T. and Mitsuyama, M. **(2001).** Difference in cholesterol-binding and cytolytic activities in between listeriolysin O nad seeligerolysin O: apossible role of alanine residue in tryptophan-rich undecapeptide. FEMS Microbiol. Lett. **203**,185-189.

Jaradat, Z.W., Schutze, G.E. and Bhunia, A.K. **(2002)**. Genetic homogeneity among *Listeria monoctyogenes* strains from infected patients and meat products from two geographic locations determined by phenotyping, ribotyping and PCR analysis of virulence genes. Int. Food Microbiol. **76**, 1-10.

Jaton, K., Sahli, R. and Bille, J. **(1992)**. Development of polymerase chain reaction assay for detection of *Listeria monocytogenes* in clinical cerebrospinal fluid samples. J. Clin. Microbiol. **30**, 1931-1936.

Jeyashekaran, G., Karunasagar, I. and Karunasagar, I. **(1996)**. Incidence of *Listeria* spp in tropical fish. Int. J. Food Microbiol. **31**, 333-340.

Johnson, S., Dewitt, W., Swaminathan, B. and Wenger, J. **(1990).** Reactivity profile of a monoclonal antibody to β-hemolysin of *Listeria monocytogenes* serotype 4b. Abstr. Annu. Meet. Am. SOC. Microbiol*.* **B-169**, 54.

Joosten 2000

Jothikumar, N., Wang, X. and Griffiths, M.W. **(2003)**. Real-time multiplex SYBR green I based PCR assay for the simultaneous detection of salmonella the simultaneous detection of salmonella serovars and *Listeria monocytogenes.* J. Food. protect. **66**, 2141-2145.

Jung, Y.S., Frank, J.F., Brackett, R.E. and Chen, J. **(2003)**. Polymerase chain reaction detection of *Listeria monocytogenes* on frankfurters using oligonucleotide primers targeting the genes encoding internalin AB. J. Food Protect. **66,** 237-241.

Kamat,A.S. and Nair,P.M. **(1994)**. Incidence of *Listeria* species in Indian sea foods and meat. J. Food Safety **14**, 117-130.

Karunasagar, I. and Karunasagar, I. **(2000)**. Listeria in tropical fish and fishery products. Int. J. Food Microbiol. **62**, 177-181.

Kathariou, S., Metz, P., Hof, H., Goebel, W. **(1987)**. Tn916-induced mutations in hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bactiol. **169**,1291-1297.

Kathariou, S., Pine, L., George, V., Carlone, G. M. and Holloway, B. P. **(1990)**. Nonhemolytic *Listeria monocytogenes* mutants that are also noninvasive for mammalian cells in culture: evidence for coordinate regulation of virulence. Infect. Immun*.* **58**, 3988.

Kayal, S., Lilienbaum, A., Poyart, C., Memet, S., Israel, A. and Berche, P. **(1999).** Listeriolysin O dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-kB and upregulation of adhesion molecules and chemokines. Mol. Microb. **31**, 1709-1722.

Khun, M. and Goebel, W. **(1989)**. Identification of extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. immunol. **57**, 55-61.

Kim, S.H., Park, M.K., Kim, J.Y., Chuong, P.C., Lee, Y.S., Yoon, B.S., Hwang, K.K. and Lim, Y.K. **(2005)**. Development of sandwich ELISA for the detection of *Listeria* spp. Using specific flagella antibodies. J. vet. Sci. **6**, 41-46.

Kingdon, G.C., Sword, C.P. **(1970)**. Effects of *Listeria monocytogenes* hemolysin on phagocytic cells and lysosomes. Infect. immun. **1**, 356-362.

Klein, P.G. and juneja, V.K. **(1997)**. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. Appl. Environ. Microbiol. **63**, 4441-4448

Knight, M.T., Newman, M.C., Benzinger Jr., M.J., Agin, J.R., Ash, M., Sims, P. and Hughes, D. **(1996).** TECRA *Listeria* visual immunoassay (TLVIA) for detection of *Listeria* in foods: collaborative study. J. AOAC Int. **79**, 1083–1094.

Kock, C., Hello, R., Gounon, P., Ohayon, H. & Cossart, P. **(1993).** Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. J.Cell Sci. **105**, 699-710.

Kocks,C., Goin, E., Tabouret,M., Berche,P., Ohaon,H., Cossart,P. **(1992)**. *L.monocytogenes* induced actin assembly requires the actA gene product, a scuface protein. Cell **68**, 521-531.

Koo, K., and Jaykus, L.A. **(2003)**. Detection of *Listeria monocytogenes* from a model food by fluorescence resonance energy transfer based PCR with an asymmetric fluoregenic probe set. Appl. Environ. Microbiol. **69**, 1082-1088.

Kreft, J., Funke, D., Hass, A., Lottspeich, F. and Goebel, W. **(1989).** Production, purification and characterization of hemolysins from *Listeria ivanovii* and *Listeria monocytogenes* Sv4b. FEMS Microbiol. Lett. **57**, 197.

Kuhn, M. and Goebel, W. **(1989)**. Identification of extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. Infect. immun. **57**, 55

Kurz, C.L. and Ewbank, J.J. **(2003)**. *Caenorhabtidis eligans*: an emerging genetic model for the study of innate immunity. Nat. rev. Genet. **4**, 380-390.

Laemelli, U.K. **(1970).** The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. Nature **227**, 680-685.
Lamont, R.J., Postlewaite, R., and Mac Gowan, A.P., **(1988)**. *Listeria monocytogenes* and its role in human infection. Infection **17,** 7-28.

Lampel, K.A., Orlandi, P.A., Korngay, L., **(2000)**. Improved template preparation for PCR assays for detection of food borne bacterial pathogens. Appl. Environ. Microbiol. **66**, 4539-4542.

Lampidis, R., Gross, R., Sokoovic, Z., Goebel, W., and kreft, J. **(1994)**. The virulence regulator protein of listeria ivanovii is highly homologous to prfA from *Listeria monocytogenes* and both belong to the Crp-Fnr family of transcriptional regators. Mol. Microbiol. **13**, 141-151.

Lantz, P.G., Tjerneld, F., Hahn-Hagerdal, B. and Radstrom, P. **(1996).** Use of aqueous two-phase systems in sample preparation for polymerase chain reaction-based detection of microorganisms. J. Chromatogr. B: Biomed. Appl. **680**, 165–170.

Lawrence, L. M. and Gilmour, A. **(1994)**. Incidence of *Listeria* spp., *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. Appl. Environ. Micribiol. **60**, 4600-4604.

Lecuit, M., Ohayon, H., Gounon, P., Mege, RM., Cossart, P. **(1997)**. Internalin of *Listeria monocytogenes* with an intact leucinerich repeat region is sufficient to promote internalization. Infect. immunol. **65**, 5309-5319.

Lehner, A., Loncarevic, S., Wagner, M., Kreike, J., Brandl, E. **(1999)**. A rapid differentiation of *Listeria monocytogenes* by use of PCR SSCP in the listeriolysin O (hlyA) locus. J. Microbiol. Methods. **34**, 165-171.

Leimister-Wachter, M. and Chakraborty, T. **(1989).** Detection of Listeriolysin the thiol dependent hemolysin in *Listeria monocytogenes*, *Listeria* innocua and *Listeria* seeligeri. Infect. Immune **57**, 2350-2357.

Lhopital, S., Marly, J., Pardon, P. and Berche, P., **(1993).** Kinetics of antibody production against listeriolysin O in sheep with listeriosis. J. clin. microbial. 31, 1537-1540.

Li, X., Bodjellab, N. and Zhao, X. **(2000)**. Combined PCR and slot blot assay for detection of Salmonella and *Listeria monocytogenes*. Int. J. Food Microbiol. **56**,167-177.

Lingnau, A., Domann, E., Hudel, M., Bock, M., Nichterlein, T., Wehland, J. and Chakraborthy, T. **(1995)**. Expression of *Listeria monocytogenes* EGD inlA and inlB genes, whose products mediate bacterial entry into tissue culture cell lines, b PrfA-dependent and independentent mechanisms. Infect.Immun. **63**, 3896.

Liu, D. **(2004).** *Listeria monocytogenes* comparitive interpretation of mouse virulence assay. FEMS Microbiol. **233**, 159-164.

Lorber, B. **(2005)**. *Listeria monocytogenes* in : Mandell, G.L., Bennett, J.E., Dolin, R., Man, Douglas. and Brennett's principles and practice of infectious diseases. (Ed 6). Philidelphia: Elsevier Churchil Livingstone, **2**, 2478-2484.

Low, J.C, Davies, R.C. and Donachie, W. **(1992)**. Purification of Listeriolysin O and development of Immunoassay for the diagnosis of Listeria infection in sheep. J. Clin. Microbiol. , 2705-2708.

Lubeck, P.S., and Hoorfar, J. **(2003)**. PCR technology and applications to zoonotic food-borne bacterial pathogens. Methods Mol. Biol. **216**, 65-84.

Lunge, V.R., Miller, B.J., Livak, K.J., Batt, C.A. **(2002)**. Factors affecting the performance of 5' nuclease PCR assays for *Listeria monocytogenes* detection. J. Microbiol. Methods **51**, 361–368.

Mackaness, G. B. **(1962).** Cellular resistance to infection. J. Exp. Med. **116**, 381.

Mandal, M. and Lee, K.D. **(2002)**. Listeriolysin O liposome mediated cytolytic delivery of macromolecule antigen in vivo: enhancement of antigen-specific cytotoxic T lymphocyte frequency, activity, and tumor production. Biochemical et. Biophysica acta **1563**, 7-17.

Mansfield, B.E., Dionne, M.S., Schneider, d.S. and Freitag, N.E. **(2003).** Exploitation of host pathogen interactions using *Listeria monocytogenes* and *Drosophila melanogaster*. Cell microbial. **5**, 901-911.

Marth, E.H. **(1988)**. Disease characteristics of *Listeria monocytogenes*. Food Technol. **42,** 165-168.

Mattingly, J.A., Butman, B.T., Plank, M.C., Durham, R.J. and Robison, B.J. **(1988).** Rapid monoclonal antibody-based enzymelinked immunosorbent assay for detection of Listeria in food products. J. Assoc. Off. Anal. Chem. **71**, 679–681.

Mc Lauchlin, J., Mitchell, R.T., Smerdon, W.J. and Jewell, K. **(2004)**. *Listeria monocytogenes* and listeriosis: a review of hazard characterization for the use in microbial assessment of foods. Int. J. Food Microbiol. **92**, 15-33.

McKellar, R.C. **(1994).** Use of the CAMP Test for Identification of *Listeria monocytogenes.* Appl. Environ. microbial. **60**, 4219-4225.

Mead, P.S., Sluskar, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., and Tauxe, R.V. **(1999)**. Food related illness and death in the United States. Emerg. Infect. Dis. **5**, 607-625.

Meng, J., Doyle, M.P., **(2002)**. Introduction: micribiological food safety. Microb. Infect. **4**, 395-397.

Mengaud, J., Vicente, M.F., Chenevert, J., Pereira, J.M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Dim, J.C. and Cossart, P. **(1988).** Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56**, 766.

Monack, D.M., Theriot, J.A. **(2001)**. Actin based motility is sufficient for bacterial membrane perturbation formation and host cell uptake. Cell microbiol. **3**, 633-647

Murray, E.G.D., Webb, R.A. and Swann, M.B.R. **(1926)** A disease of rabbits characterized by large mononuclear leucoctosis caused b a hitherto undiscribed bacillus Bacterium monocytogenes(n.sp.) . J. Pathol. Bacteriol. **29**, 404.

Mylonakis, E., Paliou, M. and Hohmann, E.L. **(2002)**. Listeriosis during pregnancy: a series of review of 222 cases. Medicine **81**, 260-269.

Nato, F., Reich, K., Lhopital, S., Rouyre, S., Geoffroy, C., Mazie, J. C. and Cossart, P. **(1991).** Production and characterization of neutralizing and nonneutralizing monoclonal antibodies against listeriolysin O. Infect. Immun*.* **59**, 4641.

Needham, A.J., Kibart, M., Crossley, H., Ingham, P.W. and Foster, S.J. **(2004)**, *Drosophila melanogaster* as a model host for *Staphylococcus aureus* infection. Microbiol. **150**, 2347-2355.

Neely, M.N., Pfeifer, J.D. and caparon, M. **(2002).** *Streptococcus Zebrafish* model of bacterial pathogenesis. Infect. Immun. **70**, 3904-3914.

Neiderhauser, C., Luthy, J. and Chandrain, U. **(1994)**. Direct detection of *Listeria monocytogenes* using para magnetic bead DNA extraction and enzymatic DNA amplification. Mol. Cell Probes **8,** 223-228.

Niebuhr, K., Chakraborty, T., Rohde, M., Gazlig, T., Jansen, B., Kollner, P. and Wehland, J. **(1993)**. Localization of the Act A poly-peptide of *Listeria monocytogenes* in infected tissue culture cell lines: Act A is not associated with actin "comets". Infect. Immun. **61**, 2793.

Nomura, T., Kawamura, I., Tscuchiya, K., Kohda, C., Baba, H., Ito, Y., Kimoto, T., Watanabe, I. and Mitsuyama, M. **(2002).** Essential role of interleukin-12(IL-12) and IL-18 for gamma interferon production induced by listeriolysin O in mouse spleen cells. Infect. Immunol. , 1049-1055.

Norton, D.M., McCamey, M.A., Gall, K.L., Scarlett, J.M., Boor, K.J. and Weidmann, M. **(2001)**. Molecular studies on the ecology of Listeria monocytogenes in the smoked fish processing industry. Appl environ microbiol **67**, 198-205.

Norton, D.M., Scarlett, J.M., Horton, K., Se, D., Thimothe, J., Boor, K.J. and Wiedmann, M. **(2001)**. Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked food industry. Appl. Environ. Microbiol. **67**, 646-653.

Notermans, S.H., Dufrenne, J., Leimeister-Wachter, M., Domann, E. and Chakraborty, T. **(1991)**. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* species. Appl. Environ. Microbiol. **57**,2666-2670.

Nyfeldt, A. **(1929)**. Etiologie de la mononucleose infectieuse. Compt. Rend. Soc. Biol. **101**, 590.

O'Connor,L., Joy,J., Kane,M., Smith,T. and Maher,M.(2000). Rapid polymerase chain reaction reaction/DNA probe membrane based assay for the detection of Listeria and Listeria monocytogenes on food. J.Food Protect. 63, 337-342

Olcen, P., Lantz, P.G. and Backman, A. **(1995)**. Rapid diagnosis of bacterial meningitis by a seminested PCR strategy. Scand. J. Infect. Dis. **27**, 537-539.

Paillard, D., Dubois, V., Duran, R., Nathier, F., Guittet, C., Caumette, P. and Qentin, C. **(2003)**. Rapid identification of *Listeria* species by using restriction fragment by polymorhism of PCR amplified 23S rRNA gene fragments. Appl. Environ. Microbiol. **69**, 6386-6392.

Palmer, M. **(2001)**. The family of thiol activated, cholesterol binding cytolysins. Toxicon. **39**, 1681-1689.

Pangallo, D., Kaclikova, E., Kuchta, T. and Drahovska, H. **(2001)**. Detection of *Listeria monocytogenes* by polymerase chain reaction oriented to inl B gene. New Microbiol. **24,** 333-339.

Paoli, G.C., Chen, C.Y. and Brewster, J.D. **(2004).** Single-chain Fv antibody with specificity for *Listeria monocytogenes.* J. Immunol. Methods **289**, 147-155.

Parrisius, J., Bhakdi, S., Roth, M., Tranum-Jensen, J., Goebel, W. and Seeliger, H.P.R. **(1986).** Production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes*. Infect. Immun*.* **51**, 314.

Pearson, L.J. and Marth, E. H. **(1990)**. *Listeria monocytogenes* – threat to a safe food supply: a review. J. Dairy Sci. **73**, 912-928.

Pierie, J.H.H. **(1927)**. A new disease of wild rodents. "Tiger River Disease". Pub. S. Afr. Inst. Med. Res. **3**,163.

Pingulkar, K.,Kamat, A. and Bongirwar,D. **(2001)**. Microbiological quality of fresh leafy vegetables, salad components and ready to eat salads: an evidence of inhibition of *Listeria monocytogenes* in tomatoes. Int. J. food. Sci. nutr. **52,** 15-23.

Racz,P., Tenner,K. and Mero,E.(1972). Experimental Listeria enteritis. I An electron microscopic study of the epithelial phase in experimental Listeria infection. Lab.Invest.26,694-700

Rahme, L.G., Tan, M.W., Le, L., Wong, S.M., Tompkins, R.G., Calderwood, S.B. and Ausubel, F.M. **(1997).** Use of model plant hosts to identify *Pseudomonas aeroginosa* virulence factors. Proc. Natl. Acad. Sci. USA **94**, 13245-13250.

Rodriguez-Lazaro, D., Hernandez, M. and Pla, M. **(2004a)**. Simultaneous quantitative detection of *Listeria* spp. and *Listeria monocytogenes* using a duplex real time PCR based assay. FEMS Microbiol. Lett. **233**, 257-267.

Rudi, K., Naterstadt, K., Dromtorp, S.M. and Holo, H. **(2005)**. Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR . Lett. Appl. Microbiol. **40**, 301-306.

Ryser, E.T., Arimi, S.M., Bunduki, M.M.C. and Donnelly, C.W. **(1996)**. Recovery of different *Listeria* ribotypes from naturally contaminated, Raw refrigerated meat and poultry products with two primary enrichment media. Appl. Environ. Microbiol. **62**, 1781-1787.

Ryser,E.T. and Marth, E.H. (**1999).** *Listeria*, Listeriosis and Food Safety. Marcel Dekker , Inc. New york. P 738.

Sallen, B., Rajoharison, A., Desvarenne, S., Quinn, F. and Mabilat, C. **(1996)**. Comparitive analysis of 16S And 23S rRNA sequences of *Listeria* species. Int. J. Syst. Bacteriol. **46**, 669-674.

Sampath kumar, B., Xavier, I.J., Yu L.S.L. and Khachatourians, G.G. **(1999)**, Production of listeriolysin O by *Listeria monocytogenes* (Scott A) under heat shock conditions. Int. J. Food microbiol. **48**,131-137.

Schlech,W.F. **(2000)**. Food borne listeriosis. Clin. Infect. Disease. **31**, 770-775.

Schleiss, M.R., Bourne, N., Bravo, F.J., Jensen, N.J. and Bernstein, D.I. **(2003)**. Quantitative-competitive PCR monitering of viral load following experimental guinea pig cytomegalovirus infection. J. Virol. Methods. **108**, 103-110.

Schmid, M.R., Walchor, M., Bubert, A., Wagner, M. and Schlefer, K.H. **(2003)**. Nucleic acid-based, cultivation-independent detection of *Listeria* spp. and genotypes in *Listeria monocytogenes*. FEMS Immunol. Med. Microbiol. **35**, 215-225.

Schneider, D. **(2000)**. Using Drosophila as a model insect. Nature Rev. Genet. **1**, 218-226.

Schubert, W.D., Urbanke, C. and ziehm, T. **(2002)**. Structure of internalin, major invasion protein of *Listeria monocytogenes*, in complex with its human receptor E-cadherin. Cell **111**,825-836.

Schuchat, A., Deaver, K. and Hayes, P.S. **(1993)**. Gastrointestinal carriages of *Listeria monocytogenes* in household contacts of patients with listeriosis. J. Infect. Dis. **167**, 1261-1262.

Schuchat, A., Lizano, C. and Broome, C.V. **(1991)**. Outbreak of neonatal listeriosis associated with mineral oil by Pediatr. Infect. Dis. **10**, 183-189.

Schuerch, D., Wilson-kubalek, E.M. and Tweten, R.K. **(2005)**. Molecular basis of listeriolysin O pH dependence. PNAS **102**, 12537-12542.

Sergeev, N., Distler, M., Courtney, S., Al-Khaldi, S.F., Volokhov, D., Chizhikov, V. and Rasooly,A. **(2004)**. Multipathogen oligonucleotide microarray for environmental and biodefense applications. Bio-sens. Bioelectron. **20**, 684-698.

Sewell, A.M., Warburton, D.W., Boville, A., Daley, E.F. and Mullen, K. **(2003)**. The development of an efficient and rapid enzyme linked fluorescence assay method for the detection of *Listeria* spp. from foods. Int. J. Food Microbiol. **81**,123-129.

Silbernagel, K., Jechorek, R., Kaufer, A.L. and Johnson, R.L. **(2005).** Evaluation of the VIDAS *Listeria* immunoassay for the detetion of *Listeria* in foods using Demi–Fraser and Fraser enrichment broths, as modification of AOAC Official Method 999.06 (AOAC Official Method 2004.06), J. AOAC Int. **88,** 750–760.

Skjerve, E., Bos, W. and van der Gaag, B. **(1991).** Evaluation of monoclonal antibodies to *Listeria monocytogenes* flagella by checker board ELISA and cluster analysis. J. Immunol. Methods **144**, 11–17.

Sneath, P.H.A., Mair, N.S., Sharpe, N. E. and Holt, J.G. (1986). Bergey's Manual of Systematic Bacteriology, vol. 2, Williams and Wilkins Co., Baltimore. pp

Solomon, J.M., Rupper, A., Cardelli, J.A. and Isberg, R.R. **(2000).** Intracellular growth of *Legionella pnemophila* in *Dictyostelium Discoideum*, a system for genetic analysis of host pathogen Interactions. Infect. Immun. **68**, 2939-2947.

Srivastava, S., Sen, M.R., Kumar, A. and Anupurba, S. **(2005)**. Neonatal Listerosis I. J. Pediatr. **72**, 1059-1060.

Steinmetz, I., Rheinheimer, C. and bitter-Suermann, D. **(1992).** Rapid identification of Ligonellae by colony blot assay based on a genus –specific monoclonal antibody. J. clin. Microbial. , 1016-1018.

Sun, A.N., Camilli, A. and Portno, D.A. **(1990)**. Isolation of *Listeria monocytogenes* small plaque mutants defective for intracellular growth and cell to cell spread. Infect. immunol. **58**, 3770-3778.

Tappero and The Listeriosis Study Group. (1995) Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts?, 273 (14)

Tauxe, R.V. **(1997).** Emerging food borne diseases: an evolving public health challenge. Emerging Infectious Diseases **3**, 425-434.

Thimothe, J., Nigthingale, K.K., Gall, K., Scott, V.N., Weidmann, M. **(2004)**. Tracking of *Listeria monocytogenes* in smoked fish processing plants. J. Food Prot. **67**, 328-341.

Tiissen, P. **(1985).** Practice and Theory of Enzyme Immunoassays. Amsterdam : Elsevier.

Torensma, R., Visser, M.J., Aarsman, C.J.M., Poppelier, M.J.,J.G., Fluit, A.D.C. and Verhoef, J. **(1993).** Monoclonal antibodies that react with Live *Listeria* spp. 2713-2716.

Traub, W. H. and Bauer, D. **(1995)**. Simplified purification of *Listeria monocytogenes* listeriolysin O and preliminary application in the enzyme-linked immunosorbent assay (ELISA). Zentralbl. Bakteriol*.* **283**, 29.

Tully, E., Hearty, S., Leonard, P. and O'Kennedy, R. **(2006)**. The development of fluorescence based immunoassays using quantum dot-labelled antibodies for the detection of *Listeria monocyrtogenes* cell surface proteins. Int. J. Bio. Macromol. **39**, 127-134.

US Department of Agriculture (2002) Microbiology Laboratory Guidebook, Chapter 8, revision 3(online)

Uyttendaele, M., Schukkink, R., Van Germen, B. and Debevere, J. **(1995)**. Detection of Camphlobacter jejuni added to foods by using a combined selective enrichment and nucleic acid sequence based amplification (NASBA). Appl. Environ. Microbiol. **61**, 1341-1347.

Van Langendonck, N., Bottreau,.E., Baill, S., Tabournet, M., Marly, J., Pardon, P. and Velge, P. **(1998)**. Tissue culture assays using Caco-2 cell line differentiate virulent from nonvirulent *Listeria monocytogenes* strains. J. Appl. Microbiol. **85**, 337-346.

Vazquez-Boand, J.A., Dominguez, L.J., Fernandez, F.G. and Suarez, G. **(1992)**. *Listeria monocytogenes* CAMP reaction. Clin. Microbiol. Rev. **5**, 3928-3935.
Veiga, E. and Cossart, P. **(2005)**. *Listeria* hijacks the ubiquitin dependent internalization of Met. Nature Cell Biol. **7**,894-900.

Volokhov, D., Rasooly, A., Chumankov, K. and Chizhikov, V. **(2002)**. Identification of *Listeria* species by microarray based assay. J. Clin. Microbiol. **40**, 4720-4728.

Walton, C.M., Catherine, H.Wu. and George, Y.Wu. **(1999)**. A method for purification of Listeriolysin O from a hypersector strain of *Listeria monocytogenes*. Protein Expression and purification **15**, 243-245.

Wan, J., King, K., Forsyth, S. and Coventry, M.J. **(2003)**. Detection of *Listeria monocytogenes* in Salmon using Probelia polymerase chain reaction system. J. Food Prot. **66**, 436-440.

Wang, X., Jothikumar, N. and Griffiths, M.W. **(2004)**. Enrichment and DNA extraction protocols for simultaneous detection of Salmonella and *Listeria monocytogenes* in raw sausage meat with multiplex real time PCR. J. Food. Protect. **67**, 189-192.

Warke, R., Kamat, A., Kamat, M. and Thomas, P. **(2000)**. Incidence of pathogenic psychrotrophs in ice creams sold in some retail outlets in Mumbai, India. Food control. **11**, 77-83.

Watkins, J. and Seath, K.P. **(1981)**. Isolation and enumeration of *Listeria monocytogenes* from sewage, sewage sludge, and river water. J. Appl. Bacteriol. **50**, 1.

Weidman, M., Bruce, J.L., Keating, C., Johnson, A.E., McDonough, P.L. and Batt, C.A. **(1997)**. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect. Immn. **65**, 2707 2716.

Weinberger, E.D. **(1984)**. Pregnancy associated depression of cell mediated immunity. Infect. Dis. **6**, 814-831.

Weis, J. and Seeliger. **(1975)**. Incidence of *Listeria monocytogenes* in nature. Appl. Bacteriol. **50**, 1.

Welshimer, H.J. **(1960)**. Survival of *Listeria monocytogenes* in soil. J. Bacteriol. **80**, 316

Welshimer, H.J. **(1968)**. Isolation of *Listeria monocytogenes* from vegetation. J. Bacteriol. **95**, 300

Welshmer, H.J. **(1981)**. The genus *Listeria* and related organisms. In: The prokarotes: A hand book on habitats, Isolation, and identification of bacteria. 1680-1689.

Wesley, I.V., Harmon, K.M., Dickson, J.S. and Schwartz, A.R. **(2002)**. Application of multiplex polymerase chain reaction assay for the simultaneous confirmation of *Listeria monocytogenes* and other *Listeria* species in turkey sample surveillance. J. Food. Protect. **65**, 780-785.

Wuenscher, M.D., Kohler, S., Bubert, A., Gerike, U. and Goebel, W. **(1993)**. The iap gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60,has bacteriolytic activity. J. Bacteriol. **175**, 3491

Yau, K.Y.F., Lee, H. and Hall J.C. **(2003).** Emerging trends in the synthesis and improvement of hapten-specific recombinant antibodies. Biotech. Adv. **21,** 599–637.

Zhang, C., Zhang, M., Ju, J., Nietfeidt, J., Wise, J., Terry, P.M., Olson, M., Kachman, S.D., Wiedmann, M., Samadpour, M. and Benson, A.K. **(2003)**. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: identification of segments unique to lineage II populations. J. Bacteriol. **185**, 5573-5584.