OCCURRENCE OF *LISTERIA* SPECIES IN BEEF, CHICKEN AND SEAFOODS

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DECLARATION

I hereby declare that the thesis entitled “**OCCURRENCE OF *LISTERIA* SPECIES IN BEEF, CHICKEN AND SEAFOODS”** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled "**OCCURRENCE OF *LISTERIA* SPECIES IN BEEF, CHICKEN AND SEAFOODS"** is a record of research work done independently by **ANU SUDHAKARAN (2010-13-107)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of **Dr. Anu Sudhakaran (2010-13-107)**, a candidate for the degree of **Master of Veterinary Science in Veterinary Public Health,** agree that this thesis entitled "**OCCURRENCE OF *LISTERIA* SPECIES IN BEEF, CHICKEN AND SEAFOODS**" may be submitted by **Dr. Anu Sudhakaran (2010-13-107),** in partial fulfillment of the requirement for the degree.

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Anu Sudhakaran

Dedicated To My Loving

Husband

&

Our Family

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Introduction

1. INTRODUCTION

Foodborne infections are an important public health concern worldwide and Listeriosis caused by *L.monocytogenes* is an emerging foodborne bacterial zoonosis. Because of its high case fatality rate of 20 to 30 per cent, it ranks among the most frequent cause of death due to foodborne illness. *L.monocytogenes* infections are responsible for the highest hospitalization rates (91 per cent) amongst known foodborne pathogens and have been linked to sporadic episodes and large outbreaks of human illness worldwide (Malek *et al*., 2010). Reports indicate that listeriosis has emerged to be more important in developed countries but is reported less frequently in developing countries (WHO, 1988). This could be associated with lack of awareness, lack of diagnostic facilities and limited resources together with the presence of other disease epidemics that claim more priority than listeriosis in developing countries including India.

In human beings listeriosis can manifest in invasive or non-invasive forms (Nightingale *et al.,* 2005). The invasive form is more frequently associated with well defined high risk groups including immunocompromised individuals, elderly (aged 65 years and older), pregnant women, unborn infants and neonates. The invasive form can result in still births, abortions and premature deliveries. In neonates, it can lead to septicaemia, meningitis and is often fatal, whereas in immunocompromised populations it can cause meningitis, encephalitis, meningoencephalitis and septicaemia (Bell and Kyriakides, 2005). The non invasive form results in milder food poisoning like symptoms such as fever, headache and diarrhoea, often referred to as febrile gastroenteritis (Berrada *et al*., 2006). Occupationally acquired listeriosis can occur in veterinary, laboratory and farm workers as ocular and cutaneous form.

The opportunistic *L.monocytogenes* is found widely distributed in the natural environment, thus can easily contaminate various raw and processed foods including milk and dairy products, meat, fermented sausages, fresh vegetables as well as seafood and fish products. (Yucel *et al.*, 2005). Human infection occurs mainly due to consumption of contaminated food (Minea *et al.*, 2005). First documented in 1981 (Lunden *et al.*, 2004), listeriosis outbreaks have been found to be linked to the consumption of various contaminated foods such as fresh soft cheese, cooked and frozen poultry products, pork products, meat products, hot dogs, smoked fish, vegetables, seafood, and salads (Alessandria e*t al.*, 2010) and ready-to-eat (RTE) foods stored at refrigeration temperatures that are generally consumed without heating (Gravesen *et al.*, 2000).

Currently, India ranks as the third largest exporter of beef in the world and seventh position in chicken meat export. The export of marine products from India set an ever time record of 6.1 million metric tons (Anon., 2011). Exports of products from India have been inconsistent because of the poor sanitary quality of products. Control of foodborne diseases including listeriosis represents a significant problem for the food industries, public health agencies and Government bodies.

The genus Listeria includes six different species: *L.monocytogenes, L.ivanovii, L.seeligeri, L.welshimeri, L.innocua* and *L.grayi*. *L.monocytogenes* is the most pathogenic to both man and animals. The entire infection cycle of *L.monocytogenes* infection is governed by multiple proteins such as internalin A and internalin B (encoded by *inl*A and *inl*B), hemolysin (encoded by *hly*, its product in *L.monocytogenes* being known as listeriolysin O or LLO), phosphatidylinositol-specific phospholipase C (PI-PLC, encoded by *plc*A), phosphatidylcholine-specific phospholipase C (PC-PLC, encoded by *plc*B) and actin polymerization protein (encoded by *act*A) (Jaradat *et al.*, 2002).

The minimal infective dose for listeriosis is unclear, although this is likely to vary considerably based on immunological status of individuals. The contamination level in foods associated with infection has revealed an average contamination of 10^2-10^6 cfu/g in the majority of cases (Wagner and McLauchlin, 2008). The International Commission on Microbiological Specifications for

Foods (ICMSF, 1994) recommended that the tolerance level should not be greater than 100 cfu/g in any food at the point of consumption and ‘zero tolerance’ for ready to eat foods.

A wide variety of methods are available for the detection of *Listeria* spp. from food and clinical samples. Classical bacteriological techniques are still considered to be the ‘gold standard’. But the standard culture methods for the detection and identification of listeria are laborious requiring a minimum of five days to recognize *Listeria* spp. and about ten days to identify *L.monocytogenes* by confirmatory tests and are not appropriate for routine screening of a larger number of samples. The lateral flow assay technique is a commercially available kit based on immune flow principle that allows rapid screening of samples for the presence of *L.monocytogenes*.

Advances in molecular technologies, particularly Polymerase Chain Reaction (PCR) allowed reliable microbial identification and surveillance. PCR techniques are highly sensitive and specific and enhance the likelihood of detecting *L.monocytogenes* without the need for isolating pure cultures.

Microorganisms owe their resistance to their ancestor’s ability to adapt and change. An unfortunate consequence of this process, however, is the development of microbial resistance to clinical antibiotics, food preservatives and disinfection processes. Many pathogens are developing resistance to most currently used antibiotics and their resistance gene can be transferred to other pathogenic organisms present in gastrointestinal tract (Perreten *et al.*, 1997). An undesired consequence of use of antimicrobial agents in animals is the potential development of antimicrobial-resistant zoonotic foodborne bacterial pathogens and subsequent transmission to humans as food contaminants. This process may have undesirable clinical implications within human and livestock population having contact with such resistant pathogens. *Listeria* spp. have been known to be susceptible to many antibiotics that are active against Gram positive bacteria

(Hawkins *et al.*, 1984), but more recently, reports of antibiotic resistance in *Listeria* spp. have been published (Schwaiger *et al.*, 2010).

Published information on the occurrence and distribution of *Listeria* spp. is very limited both in the veterinary and public health sectors in India. Considering all the above facts the present study was undertaken with the following objectives

1) Screening of beef, chicken and seafoods for the presence of *Listeria* spp. by culture and immunological technique

2) Comparison of conventional culture and immunological techniques for the detection of *Listeria monocytogenes*

3) Study the antibiotic sensitivity pattern of the isolates

Review of Literature

2. REVIEW OF LITERATURE

Listeriosis is one of the most important foodborne disease caused by *Listeria monocytogenes*. Meat, poultry, dairy, seafoods and vegetable products have all been implicated as vehicles of listeriosis. The main objective of this study was to evaluate the occurrence of *Listeria* spp. in various foods of animal origin.

The literature pertaining to various methods for the detection, the occurrence of *Listeria* spp. in beef, chicken and seafoods, and antibiotic sensitivity pattern has been reviewed under various headings.

2.1. DETECTION METHODS FOR *LISTERIA* SPP.

2.1.1 Conventional culture techniques

McClain and Lee (1988) developed the United States Department of Agriculture- Food Safety and Inspection Service (USDA- FSIS) method for the detection of *L. monocytogenes* from raw meat and poultry within three to four days. The method was developed specifically to detect naturally occurring *L. monocytogenes* in meat because the traditional cold enrichment procedure was extremely slow. This method could identify beta-hemolytic Listeria colonies in three to four days. The use of two-stage enrichment, highly selective Lithium chloride- Phenyl ethanol- Moxalactam (LPM) agar, and a thin-layer horse blood agar plates for the detection of beta-hemolytic Listeria isolates were the important steps of this method.

A comparative study of the Food and Drug Administration (FDA) and USDA methods for the detection of *L.monocytogenes* in foods was done by Warburton *et al.* (1991). Nineteen laboratories across Canada took part in it and the results showed that the enrichment period of the FDA method can be shortened from seven to two days without substantially reducing the number of positive samples and the USDA method proved to be slightly more efficient in isolating *L. monocytogenes* than the FDA method. They also recommended the use of Oxford agar and LPM agar than modified McBride's agar in isolating this microorganism.

Curtis and Lee (1995) compared different media and methods for the isolation of *L.monocytogenes* and reported that no single method can be recommended for all situations. They pointed out that the choice of media and methods were governed by the type of sample, number and nature of competing flora and cost.

Jemmi *et al.* (2002) carried out a prevalence study of *L.monocytogenes* in imported and exported fish and meat products in Switzerland over a nine year period (1992- 2000). Qualitative detection of *L.monocytogenes* was done by selective enrichment in University of Vermont Medium I (UVM I) and UVM II followed by plating on Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar and Oxford agar. The presumptive *Listeria* colonies were confirmed by Gram staining and catalase test, and differentiated to the species level using beta hemolysis, Christie Atkins Munch Peterson (CAMP) factor and fermentation of rhamnose. They were able to identify *L.monocytogenes* in 282 specimens out of 2217 samples screened.

Dhanashree *et al.* (2003a) adopted a modification of the USDA and FDA method for the isolation of *Listeria* spp. The procedure consisted of a pre enrichment step in Pre Enrichment Broth (PEB), followed by primary selective enrichment in UVM I and a secondary enrichment step in Modified Fraser broth. The samples were then streaked on Oxford and PALCAM agar. *L.monocytogenes* was recovered from two out of 320 food samples screened.

Panda and Garg (2003) followed the two stage enrichment procedure using UVM I and II followed by plating on Dominguez- Rodriguez isolation agar (DRIA) for isolation of *Listeria* spp. Out of 330 samples screened, *Listeria* spp. were detected in 34 samples and two was positive for *L.monocytogenes*.

Cetinkaya *et al.* (2004) conducted a prevalence study of *Listeria* spp. in chicken at the retail level. They carried out isolation by direct plating on Oxford agar after homogenizing the sample in 0.1 per cent peptone water. The samples were also used for selective enrichment in tryptone soy broth supplemented with 0.6 per cent yeast extract for 48 h at 30 ^{0}C and then plating on Oxford agar. By direct plating, *Listeria* spp. were recovered from 19 per cent of samples whereas after selective enrichment procedure organism recovered from 31 per cent of samples.

A survey conducted by Gudbjornsdottir *et al.* (2004) screened 2522 samples, revealed the incidence of *Listeria* spp. in Nordic countries. Two methods used for the isolation were Nordic committee on food analysis (NKML) method and USDA method. The USDA pre enrichment medium was UVM modified enrichment broth with nalidixic acid and acriflavin and the enrichment medium was UVM modified enrichment broth with 0.3 per cent lithium chloride, acriflavin and ferric ammonium citrate. The NKML pre enrichment medium was LB I Listeria enrichment broth base with supplements nalidixic acid, acriflavin and cycloheximide. The NKML enrichment medium was LB II Listeria enrichment broth containing nalidixic acid and acriflavin. Selective plating in both cases was done on Oxford agar. The average incidence in raw products was 15.6 per cent for meat, 22.2 per cent for poultry and 39 per cent for seafood.

Vitas and Garcia- Jalon (2004) investigated occurrence of *Listeria* spp. in fresh and processed foods in Spain. They followed L- Palcamy method (NGFIS, Netherland Government Food Inspection Service) for the isolation from meat and fish. The method used L- Palcamy broth with selective agents polymyxin 10 milligram/ litre, acriflavin HCl five milligram/ litre and ceftazidime 20 milligram/ litre. The selective plating was done on PALCAM agar. Occurrence of *Listeria* spp. in raw poultry and minced pork were 76.3 per cent and 62.3 per cent respectively.

Parihar *et al.* (2008) had carried out isolation of *Listeria* spp. from 115 raw/ fresh seafoods using a two step enrichment procedure using UVM I and UVM II followed by plating on two selective agars DRIA and PALCAM agar. The confirmation of the isolates was done using biochemical identification. They were able to recover *Listeria* spp. from 28 seafood samples and of these isolates ten were *L.monocytogenes.*

2.1.2. Immunological assays

Capita *et al*. (2001a) compared an immune assay test with USDA method for their ability to detect *Listeria* spp. on 40 chicken carcasses. When they used MOX (Modified Oxford) media for the isolation, the sensitivity of the immunoassay test was 100 per cent and the specificity 85.7 per cent, whereas the sensitivity and specificity of the PALCAM medium was 94.29 per cent and 80 per cent respectively.

Isolates of *Listeria* spp. obtained from clinical and food samples by culture method were subjected to Enzyme Linked Immuno Sorbent Assay (ELISA) test using 'PATHAALERT *Listeria* ELISA' kit to detect Listeria antigen P60 by Dhanashree *et al.* (2003b). The incidence o f *Listeria* spp. was 0.2 per cent in clinical and 17.5 per cent in food samples by both culture method and immunological technique.

Mena *et al.* (2004) studied on the incidence of *L.monocytogenes* in different food products in Portugal. The samples were analysed by mini Vitek Immuno Diagnostic Assay System *Listeria Monocytogenes* (VIDAS LMO) method, an enzyme linked fluroscent immunoassay performed in mini VIDAS instrument after primary and secondary enrichment in Fraser broth. Positive samples were confirmed by isolation on Oxford and PALCAM media. Of 1035 samples screened, seven per cent were positive for *L.monocytogenes*.

Bohaychuk *et al.* (2005) evaluated various detection methods for screening meat and poultry products for the presence of foodborne pathogens including *Listeria* spp. Rapid and molecular technologies such as ELISA, Polymerase Chain Reaction (PCR) and lateral flow immunoprecipitation were compared with conventional culture technique. The lateral flow immunoprecipitation test did not produce any false negatives but due to false positive results produced they concluded that this method would be more suitable for screening meat and poultry products for ruling out *Listeria* spp.

Singh *et al.* (2006) compared PCR and competitive ELISA (cELISA) for rapid detection of *L.monocytogenes* from foods with culture method as standard. The test revealed that PCR and cultural methods were able to detect the organism from samples spiked with minimum of 10 cells/ g after 36 h incubation time whereas cELISA required 48 h incubation for the same result.

Blazkova *et al*. (2009) developed a Nucleic Acid Lateral Flow Immunoassay (NALFIA) for simultaneous detection of *Listeria* spp. and *L.monocytogenes* in food samples. The developed procedure consisted of an enrichment step (24 h), isolation of template DNA and a PCR amplification step using specific sequences (3.5 h) and an immunochromatographic detection of amplified product by NALFIA (5-15 min). The combination of nucleic acid amplification and an immunochemical based detection principle offered favourable advantage in terms of sensitivity, specificity, cost and speed.

Meyer *et al*. (2011) screened raw meat and by products (985 samples) obtained from beef and pork using the VIDAS LMO2 automated enzyme linked immunoassay method. The samples were first screened by the VIDAS LIS reagent strip for the presence of *Listeria* spp. followed by testing for the presence of *L.monocytogenes* using VIDAS LMO2. Fourteen per cent of the samples were positive for Listeria and four per cent tested positive for *L.monocytogenes*, of which three per cent were confirmed on selective agar. The samples strongly positive for *Listeria* spp. by VIDAS were positive for *L.monocytogenes*. The VIDAS system was shown to be a suitable method for screening out Listeria negative samples with a reduced assay time.

A capture ELISA for the identification of *L.monocytogenes* in food was used by the Portanti *et al.* (2011). The assay was refined by analyzing the samples of meat, seafood, dairy products, pasta and flour. The method was found to be 100 per cent specific for *L. monocytogenes* tested with a detection limit of 6.6×10^3 colony forming units (cfu)/ ml. Among the food samples analysed using L. mono capture ELISA, 51 were found positive for *L. monocytogenes* and 22 negative. Analysis of the sample with standard microbiological methods also revealed the same results.

2**.1.3. Polymerase Chain Reaction**

Furrer *et al.* (1991) used PCR to detect *L.monocytogenes* from foods by amplifying two specific DNA fragments of α and β hemolysin genes. In the analysis six out of 50 samples of cooked sausage products were found to be contaminated with *Listeria* spp. by conventional culture techniques. Out of these six isolates, three were confirmed as *L.monocytogenes* by PCR technique.

Bansal *et al.* (1996) developed a multiplex PCR assay for the routine detection of Listeria in food. The assay employed a short culture enrichment step using primary enrichment broth followed by isolation of bacterial cells and detection by Multiplex PCR. A multiplex of synthetic oligonucleotide primers was used in the PCR assay, which comprised genus-specific primers complementary to ribosomal DNA sequence for detecting all *Listeria* species and species-specific primers complementary to the nucleotide sequence of the virulence gene listeriolysin O, for the detection of *L. monocytogenes.* Out of the 350 food samples tested by the PCR procedure, 28 per cent contained *Listeria* spp. and 16 per cent were positive for *L.monocytogenes*. When compared with standard cultural procedures no false positive or false negative results were obtained with PCR assay and the results obtained within 48 h of sampling.

Simon *et al.* (1996) carried out different protocols for DNA extraction and PCR for the detection of *L.monocytogenes* from cold smoked salmon. Smoked salmon contains phenolics, cresols and aldehydes which are potential inhibitors of the PCR which can be surmounted either by removing the inhibiting substances or by the addition of a compound which enhance the reaction. The DNA extraction protocol using hexadecyl trimethyl ammonium bromide with ether separation or column purification was effective in removal of the inhibitors of PCR present in artificially inoculated food samples. A nested PCR detection protocol employing the *prf* A gene involved in the regulation of listeriolysin synthesis as the target was designed and used for the analysis.

Dhanashree *et al.* (2003a) confirmed *L.monocytogenes* isolates obtained from clinical and food samples by PCR using the primer pairs Unilis A- Lis 1B, Mono A- Mono B and Unilis A- Mono B. *L.monocytogenes* was isolated from two each of clinical and food samples from screened 633 clinical and 320 food samples respectively.

Rodriguez- Lazaro *et al*. (2004) developed and assessed real-time PCR (RTi-PCR) assays for the detection and quantification of the foodborne pathogen *L. monocytogenes*. The target genes were *hly* and *iap*. The assays were 100 per cent specific, as determined with 100 Listeria strains and 45 non-Listeria strains, and highly sensitive, with detection limits of one target molecule in 11 to 56 per cent of the reactions with purified DNA and three colony forming units in 56 to 89 per cent of the reactions with bacterial suspensions. The *hly*-based assay accurately quantified *L. monocytogenes* in all of the samples tested. The *iap*-based assay, in contrast, was unsuitable for quantification purposes, underestimating the bacterial counts by three to four log units in a significant proportion of the samples due to serovar related target sequence variability. The combination of the two assays enabled us to classify *L. monocytogenes* isolates into one of the two major phylogenetic divisions of the species, I and II.

Gouws and Liedemann (2005) evaluated diagnostic PCR for the detection of *L.monocytogenes* in food products. Of the 27 food samples tested, 74 per cent were presumptively positive for Listeria on Oxford agar, while 44 per cent were presumptively positive for *L. monocytogenes* on RAPID'L. mono. Only 37 per cent of samples were confirmed to be positive for *L. monocytogenes* by PCR amplification of the *hly* gene (732 bp). PCR was able to eliminate the false positives and detect all *L. monocytogenes* in the food products, unlike the conventional methods used in the food industry.

Amagliani *et al*. (2007) evaluated a commercial test kit for the detection of *L.monocytogenes* by PCR, using different DNA extraction methods. For their study food samples were spiked with known concentrations of *L. monocytogenes* and culture-enriched for 24 h. DNA extracted using three commercial kits and two standard methods, was amplified in species specific PCR kit for *L.monocytogenes*. They proved that the PCR-based method to be a reliable means of detecting the pathogen in food samples independently from the extraction procedure used, even for a contamination cell number of one cfu/g before culture enrichment.

A rapid method for the detection of *L monocytogenes* in foods combining culture enrichment and real-time PCR was compared to the ISO 11290-1 standard method by Grady *et al.* (2009). The culture enrichment component of the rapid method was based on the ISO standard and includes 24 hour incubation in half-Fraser broth, four hour incubation in Fraser broth followed by DNA extraction and real-time PCR detection of the *ssrA* gene of *L. monocytogenes*. The method has a limit of detection of one to five cfu/25 g of food sample and can be performed in two working days compared to up to seven days for the ISO standard. A variety of food samples from retail outlets and food processing plants (175) and controls (31) were tested using rapid and conventional methods. The rapid method was 99.44 per cent specific, 96.15 per cent sensitive and 99.03 per cent accurate when compared to the standard method.

Gawade *et al*. (2010) carried out isolation and confirmation of *Listeria* spp. from seafood off Goa region by PCR. In their study, 111 marine fish samples including prawns, finfishes and bivalves were screened for the presence of *Listeria* species. The isolates were characterized biochemically and further *L. monocytogenes* were confirmed by polymerase chain reaction (PCR) technique using the *hlyA* gene as a tool to differentiate between *L. monocytogenes* and other non-pathogenic *Listeria* species. Out of 111 samples five (4.5 per cent) samples were positive for *L. monocytogenes*.

Lakicevic *et al.* (2010) carried out identification of *Listeria* spp. by culture methods, PCR and by direct sequencing of 16s RNA and nucleotide analysis. The samples were amplified for PCR with primers complementary to the *hly*A gene and the *iap* gene. *L.monocytogenes* was confirmed in two out of 141 samples by all methods. One discordant sample was misidentified as *L. monocytogenes* by PCR assay by using primers complementary to the *iap* gene was *L.innocua* by the culture method, thus underscoring the biases of traditional methods. The results obtained by PCR using *iap* gene showed that the other discordant sample, misidentified by culture method as *L.welshimeri*, belonged to *L.innocua*. These results underscore the reliability and accuracy of the PCR method. The ambiguous identification was resolved by direct sequencing of the 16S rRNA and nucleotide sequence analysis revealed homology with *L.innocua* (92 per cent). The obtained results suggested the need to use combined methods in order to obtain accurate identification of *Listeria* spp.

2.2. OCCURRENCE OF *LISTERIA* SPECIES IN FOOD SAMPLES

2.2.1. Beef

McClain and Lee (1988) could recover *L. monocytogenes* from 20 out of 41 samples of frozen ground beef by the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) method for isolation of *L. monocytogenes* from raw meat and poultry in three to four days.

Rahmat *et al.* (1991) compared the prevalence of *L.monocytogenes* in imported and local beef collected from four wet markets namely A, B, C and D and two supermarkets (E and F). Four samples each of imported and local beef were collected from wet markets and two each of imported and local beef from supermarkets. None of the samples from supermarkets were positive for *L.monocytogenes*. 50 per cent of the imported beef samples obtained from markets B and D were positive while only 25 per cent of the imported beef samples from markets A and C were positive. As for local beef, 50 per cent of the samples obtained from market B were positive while only 25 per cent of the samples obtained from market A were positive. All local beef samples tested from markets C and D were negative for *L. monocytogenes.*

A total of 234 samples of food consisting of 158 raw and 76 ready to eat samples were examined by Arumugaswamy *et al*. (1994) for the presence of *L. monocytogenes*. The frequency of *L. monocytogenes* contamination in raw beef was 50 per cent and ready to eat meat samples were negative for the organism.

Bansal *et al.* (1996) tested eight samples of roasted beef by the PCR and reported that three samples were positive for *Listeria* spp. and among that two samples were positive for *L.monocytogenes.*

A survey was performed in 1994 and 1995 in retail foods in Denmark by Norrung *et al.* (1999) for detecting level of *L. monocytogenes*. Incidence of *L.monocytogenes* in raw meat, preserved meat products (non heat treated) and preserved heat treated meat products were 30.9 per cent, 23.5 per cent and 5 per cent respectively.

Peng and Shelef (2000) developed a method for rapid detection of low levels of Listeria in foods and next day confirmation of *L.monocytogenes*. It consisted of a six hour pre-enrichment step followed by overnight incubation in selective broth at 35 ^{0}C. Twenty five retail food samples were tested. These included three samples of milk, two eggs, and ten each of ready to eat meat and fresh ground meat. Four samples were identified as positive. Of these four, only one ground beef sample was confirmed as positive for *L. monocytogenes* by PCR.

Jemmi *et al*. (2002) studied the prevalence and risk factors for contamination with *L. monocytogenes* of imported and exported meat and fish products in Switzerland, 1992–2000. Incidence of *L.monocytogenes* in 28 samples of raw meat, 255 samples of cooked and cured meat products and 132 samples of cured and dried meat products were eleven per cent, six per cent and three per cent respectively. The risk of Listeria contamination was mainly associated with the product category and the production plant whereas the country of origin, sampling site or season had no detectable influence.

In a study carried out by Dhanashree *et al*. (2003a) to determine incidence of *Listeria* spp. in food samples collected from retail outlets in Mangalore during the period from March, 1997 to December, 2001 included fresh, dry and smoked seafood, dairy products, fresh meat and vegetable samples. When screened ten samples of beef only one (10 per cent) was positive for *L.innocua* and all samples were negative for other *Listeria* spp.

A total of 2522 samples, including swabs from processing lines and environment, personnel, raw materials and products (raw and ready-to-eat) were analysed by Gudbjornsdottir *et al*. (2004) for the presence of *L. monocytogenes* from six meat, five seafood and two poultry processing plants. The overall incidence of *L. monocytogenes* in meat processing plants varied from 0 to 15.1 per cent. Overall frequency of *Listeria* spp. and *L. monocytogenes* in 1031 meat processing plant samples were 137 (13.3 per cent) and 49 (4.8 per cent) respectively. The per cent of the raw material samples contaminated with *Listeria* spp. were 31.4 and among that 2.9 per cent with *L.monocytogenes*. Analysis showed that 37.8 per cent of the total final raw products were contaminated with

Listeria spp. and 15.6 per cent with *L.monocytogenes*. Of 43 ready to eat products examined only one (2.3 per cent) was contaminated with *L.monocytogenes*.

Mena *et al*. (2004) screened 1035 different food products commercialized in Portugal. The incidence of *L. monocytogenes* was 17.7 per cent in raw meat samples.

Molla *et al.* (2004) carried out a cross sectional study from September 2003 to April 2004 in 316 food samples randomly selected and purchased from retail supermarkets and other shops in Addis Ababa, Ethiopia. A total of 316 food samples consisting of different food products including 61 minced beef were assessed. The level of contamination of *Listeria* spp. of minced beef was 47.5 per cent. *L. monocytogenes* was detected in 3.44 per cent of beef samples. In addition to *L.monocytogenes*, other *Listeria* species identified were *L.innocua* (72.4 per cent), *L.welshimeri* (10.34 per cent), *L.murrayi* (6.9 per cent), *L.ivanovii* (3.44 per cent) and *L.grayi* (3.44 per cent).

In a study on occurrence of *L.monocytogenes* in fresh and processed foods in Navarra, Spain, Vitas and Garcia- Jalon (2004) screened 3685 samples. Out of 295 raw meat samples 184 contained one or more species of Listeria. *L.monocytogenes* was present in 103 samples. Other *Listeria* species were *L.innocua* (34.9 per cent), *L.welshimeri* (15.1 per cent), and *L.seeligeri* (1.6 per cent). Occurrence of *Listeria* species in cooked meat were 18.2 per cent and 8.8 per cent samples were positive for *L.monocytogenes*. Other *Listeria* species in cooked meat were *L.innocua* (11.4 per cent) and *L.welshimeri* (1.3 per cent).

Based on the prevalence study conducted by Busani *et al.* (2005), level of *L.monocytogenes* was 2.4 per cent in 42,300 food samples screened. Contamination rate of the organism in different beef products were two to five per cent.

A study on the prevalence of *Listeria* species in meat products in Ankara, Turkey was carried out by Yucel *et al.* (2005). In this study, a total of 146 raw and cooked meat samples were analysed for the presence of *Listeria* spp. Prevalence of *Listeria* species in raw beef and cooked red meat were 73.6 per cent and 16.1 per cent respectively. *L. monocytogenes* was isolated from 5.2 per cent of raw beef samples and 6.4 per cent of cooked red meat samples. Other isolates from raw beef samples included 63.1 per cent *L.innocua* and 5.2 per cent *L.welshimeri*, whereas only 9.6 per cent of cooked meat samples were positive for *L.innocua*.

Prevalence of *L.monocytogenes* in Japan in minced beef was reported as 12 per cent by Jemmi and Stephan (2006).

Barros *et al.* (2007) studied the occurrence of *Listeria* species in beef and processing plants. Four hundred and forty three samples were collected from equipments, installations and products from 11 meat processing establishments in Parana state, Brazil and reported that 38.1 per cent of the samples were positive for *Listeria* spp. The identified species were *L.monocytogenes* (12.6 per cent), *L.innocua* (78.4 per cent), *L.seeligeri* (1.2 per cent), *L.welshimeri* (7.2 per cent) and *L.grayi* (0.6 per cent).

Hedge *et al.* (2007) screened 50 food samples collected from retail outlets using the USDA-FSIS method for the detection and isolation of *L.monocytogenes* utilizing BBL CHROM agar Listeria and Modified Oxford as selective agar. Of the fifty food samples tested, two samples were positive for *L.monocytogenes* and none of the meat products were positive for the organism.

Takahashi *et al*. (2007) attempted to isolate *L. monocytogenes* from skin, contents of large intestines and carcasses of cattle introduced to a slaughterhouse in Japan in order to identify source of contamination for this pathogen. Sixty skin samples, 60 samples of the contents of large intestines and 30 carcass samples were collected in June, August and November 2003 for the study. *Listeria* spp. and *L. monocytogenes* were isolated from 30 (50 per cent) and three (five per cent) of the cattle skin samples respectively. However, no *Listeria* spp., including *L.monocytogenes*, was isolated from intestinal contents or carcasses. Seven isolates of *L.monocytogenes* were obtained, of which five and two strains were of serotypes 1/2a and 1/2b respectively.

A survey on the Listeria contamination of ready-to-eat food products in Vienna, Austria was conducted by Wagner *et al.* (2007). *Listeria* spp. and *L.monocytogenes* were tested positive in 5.6 per cent and 1.7 per cent of beef samples analysed.

Aragon-Alegro *et al.* (2008) screened 40 samples of ground beef collected from supermarkets located in Sao Paulo city, Brazil. *Listeria* species were present in 97.5 per cent of samples and among that 67.5 per cent samples were positive for *L.monocytogenes*. Other *Listeria* species such as *L.innocua, L.seeligeri* and *L.welshimeri* were also isolated from ground beef samples.

Jalali and Abedi (2008) found out that *Listeria* spp. were present in 10.6 per cent of 66 screened beef samples from Isfahan, center of Iran and 3.03 per cent of samples confirmed *L.monocytogenes*.

Prevalence of *L. monocytogenes* in 20 raw beef samples from Greek open-air Markets was shown as 20 per cent in a study by Filiousis *et al*. (2009).

The prevalence of *L. monocytogenes* in ready-to-eat products from markets in Northern Spain was studied by Garrido *et al.* (2009). When screened 220 samples of vacuum packed deli meat products, 20 samples were found to be contaminated with *Listeria* species. They isolated *L.monocytogenes* from 2.7 per cent, *L.innocua* from 2.3 per cent, and *L.welshimeri* from 6.4 per cent of samples. Out of 200 samples of opened deli meat products 37 samples were shown contamination with *Listeria* species. They isolated *L.monocytogenes* and

L.innocua from 17 samples each. *L.seeligeri*, *L.grayi* and *L.welshimeri* were isolated from two, one and five samples respectively.

A study was undertaken by Gianfranceschi *et al*. (2009) to know the serotypes of 674 *L. monocytogenes* isolates collected from different Italian geographical areas during 2002–2005. Among these 558 isolates were from foods. When examined 68 isolates from raw meat, they confirmed serotypes 1/2a (30), 1/2b (9), 1/2c (27), 3c (1) and 4b (1). The serotypes of *L.monocytogenes* in 50 isolates from ready to eat meat were 1/2a (9), 1/2b (16), 1/2c (18), 4ab (1), 4b (4), 4e (1) and nt (1). The serotypes of 21 isolates of *L.monocytogenes* from meat processing plants were 1/2a (6), 1/2b 5) and 1/2c (10).

Mengesha *et al.* (2009) carried out a study on occurrence and distribution of *Listeria monocytogenes* and other *Listeria* species in ready-to-eat and raw meat products. Of the 711 food samples examined, 189 samples were Listeria positive of which 34 samples were *L. monocytogenes.*

Malek *et al*. (2010) conducted a study on occurrence of *Listeria* in 25 samples of minced frozen beef collected from Assiut city, Egypt. *Listeria* spp. were detected in eight (32 per cent) samples. PCR results showed that *L.monocytogenes* were present in one of imported minced frozen beef.

Pesavento *et al*. (2010) conducted a research survey to check the presence of *Listeria* spp. in raw meat and retail products. Total prevalence was 11.7 per cent (148/1268) and in beef it was 17.3 per cent (41/237). Some samples contained two or three different strains of *Listeria* species. Among the 45 isolates of *Listeria* species in beef *L.monocytogenes*, *L.innocua*, *L.welshimeri*, *L.seeligeri* and *L.ivanovii* were 11 (22.4 per cent), 19 (42.2 per cent), ten (22.2 per cent), three (6.67 per cent) and two (4.4 per cent) respectively. *L.grayi* was not detected from beef samples.

Stonsaovapak and Boonyaratanakornkit (2010) screened a total of 380 meat and meat products, dairy and dairy products, fresh vegetables, fresh seafood, and ready-to-eat food samples from supermarkets in Bangkok, Thailand. The overall incidence of *Listeria* spp. was 16.8 per cent, most of them were isolated from raw meat and vegetables. *L. monocytogenes* was isolated from 18 out of 380 samples. When 30 samples of minced beef were analysed 26.7 per cent were positive for *Listeria* species and *L.monocytogenes* were detected only in ten per cent of samples.

Vasilev *et al*. (2010) surveyed *L. monocytogenes* strains, isolated from ready-to-eat foods in Israel over a period of 10 years, 1998–2007. The level of incidence of *L.monocytogenes* in meat samples was seven per cent among 1297 samples tested.

Yan *et al*. (2010) screened a total of 2177 food samples from nine cities in northern China during 2005 to 2007 for the presence of *L. monocytogenes*. Contamination with *L. monocytogenes* was detected in 4.13 per cent of the samples representing various food products. The isolation rate in raw meat products was 6.28 per cent and in cooked meat was 3.31 per cent. Among raw meat samples, the pathogen was detected from 2.55 per cent of raw beef.

Meyer *et al*. (2011) collected 501 samples of beef consisting of meat cuts, kidney, liver, heart and tongue from European Union approved slaughter houses and processing plants in Germany. They found that 77 samples were contaminated with *Listeria* spp. using the VIDAS® LIS reagent strip. Out of the 77 positive samples, 13 were positive for *L. monocytogenes* using the VIDAS® LMO2 reagent strip whereas 12 were confirmed by the cultural method. *L. monocytogenes* could be detected from kidney, liver and meat cuts.

Bouayad and Hamdi (2012) screened 227 food samples consisting of 23 unpacked sliced meat products and 94 cooked meat dishes (sandwiches and hot dishes). *Listeria* spp. were obtained from 21 out of 227 food samples which included 2.6 per cent *L. monocytogenes*, 4.8 per cent *L.innocua*, 1.3 per cent *L.ivanovii* and 0.4 per cent *L.welshimeri*. Among the samples, only the sausage sandwiches and cooked meat dish were found contaminated with *L. monocytogenes.*

The occurrence of *Listeria* spp. and *L. monocytogenes* in retail ready to eat meat and fish products in Vancouver, British Columbia (B.C.) was investigated by Kovacevic *et al.* (2012). Conventional methods were used to recover *Listeria* spp. from 40 samples each of deli meat and fish collected from 17 stores. No *Listeria* spp. were recovered from meat samples.

2.2.2. Chicken

McClain and Lee (1988) recovered *L. monocytogenes* from seven out of 22 samples of poultry by the USDA-FSIS method for isolation of *L. monocytogenes* from raw meat and poultry in three to four days.

Pini and Gilbert (1988) reported that the prevalence of *L.monocytogenes* in fresh and frozen chicken were 46 per cent and 54 per cent respectively.

Skovgaard and Morgen (1988) found out that *L.monocytogenes* was present in 47 per cent of the screened neck and skin samples of poultry.

The prevalence of *Listeria* spp. in the skin of poultry legs, wings, and whole liver, representing 160 packages, and purchased from three supermarkets in California was investigated by Genigeorgis *et al*. (1989). Overall, *Listeria* spp., *L. monocytogenes, L. innocua, and L. welshimeri* were present in 40.6, 13.1, 26.3, and 1.3 per cent, respectively of the chicken parts. For *L. monocytogenes* the overall prevalence in the skin of wings, drumsticks, and liver was 10, 15, and 14 per cent and varied extensively with sampling day. A total of 188 samples were collected from a slaughterhouse during four visits and analyzed for the presence of *Listeria* spp. *Listeria* spp. could not be isolated from feathers, scalding tank water overflow, chiller incoming water, neck skin, whole liver after chilling, cecum and large intestine contents. *Listeria* spp. were present in 18.8 per cent of feather picker drip water, 12.5 per cent of chiller water overflow, 37.5 per cent of recycling water for cleaning gutters, 25 per cent of heart, and 31.3 per cent of mechanically deboned meat samples. The prevalence of *L. monocytogenes* in packaged liver and skin of drumsticks and wings at the end of the processing line was 33.3, 36.7, and 70.0 per cent respectively. After four days of storage of the same packages at 4 ^{0}C, *L. monocytogenes* was recovered from 40, 52, and 72 per cent of the respective products. The prevalence of *L. monocytogenes* on the hands and gloves of the persons hanging birds after chilling, cutting carcasses, and packaging parts was 20, 45.5, and 59 per cent respectively.

Rahmat *et al*. (1991) compared the prevalence of *L.monocytogenes* in local chicken collected from four wet markets namely A, B, C and D and two supermarkets (E and F). Hundred per cent and 50 per cent of chicken meat were positive for *L.monocytogenes* from supermarkets E and F respectively. Fifty per cent of the minced chicken samples obtained from supermarkets E and F were positive. In case of chicken organs, 50 per cent from supermarket F were positive whereas none from supermarket E. In chicken meat samples taken from local markets A, B, C and D, 50 per cent were positive in wet markets A, B and C and no samples were positive from D.

A total of 234 samples of food, consisting of 158 raw and 76 ready-to-eat food samples were examined by Arumugaswamy *et al*. (1994) for the presence of *L. monocytogenes*. The frequency of *L. monocytogenes* contamination in raw chicken portions, liver and gizzard were 60, 60 and 62 per cent respectively.

Lawrence and Gilmour (1994) determined incidence of *Listeria* species and *L.monocytogenes* in a poultry processing plant and in raw and cooked poultry products over 6 months period. Within the raw and cooked poultry processing environments, 46 per cent and 29 per cent of the samples contained *Listeria* spp. while 26 per cent and 15 per cent contained *L. monocytogenes* respectively. Various sites within the processing environment were found to be consistently positive for *L. monocytogenes* throughout the entire sampling period. Of the raw and cooked products tested, 91 per cent and 8 per cent samples were positive for *Listeria* spp. while 59 per cent and none contained *L. monocytogenes*, respectively.

Ng and Seah (1995) studied on incidence of *L.monocytogenes* from a variety of foods of chicken origin in Singapore. They detected that 6.4 per cent (5/78) of chicken roll were positive for *L.monocytogenes*. None of the fried chicken/ chicken parts (0/26) and chicken rice (0/71) was positive for *L.monocytogenes*.

Bansal *et al.* (1996) tested 19 samples of raw poultry and eight samples of cooked chicken by the PCR procedure. Food samples were collected at local food outlets by the Health Department's food inspectors, Australia. 47.36 per cent and 12.5 per cent samples contained *Listeria* spp. respectively in raw poultry and cooked chicken. In raw poultry eight out of nine samples were positive for *L.monocytogenes* and in cooked chicken none was positive for the organism.

The prevalence of *Listeria* spp. on the skin of hundred fresh chicken carcasses purchased from 20 retail stores in Leon was investigated using the routine test procedure by Capita *et al*. (2001b). *Listeria* spp., *L. monocytogenes*, *L. innocua*, *L.welshimeri*, *L. grayi* and *L.ivanovii* were present in 95, 32, 66, seven, four and two per cent of the samples, respectively.

A total of 2522 samples, including processing lines and environment, personnel, raw materials and products (raw and ready-to-eat), were analysed by Gudbjornsdottir *et al*. (2004) for the presence of *L. monocytogenes* from six meat, five seafood and two poultry processing plants. The overall incidence of *L. monocytogenes* in poultry processing plants varied from 20.6 to 24.1 per cent. Overall frequency of *Listeria* spp. and *L. monocytogenes* in 311 samples collected from poultry processing plant were 141 and 68 samples respectively.

Mena *et al*. (2004) screened 1035 different food products commercialized in Portugal. The incidence of *L. monocytogenes* in raw chicken was reported as 60 per cent.

In a study on occurrence of *L.monocytogenes* in fresh and processed foods in Navarra, Spain, Vitas and Garcia- Jalon (2004) screened 3685 samples. Out of 158 poultry samples 120 were positive for one or more species of Listeria. The samples positive for *L. monocytogenes*, *L.innocua*, *L.welshimeri* and *L.seeligeri* were 57 (36.1 per cent), 106 (67.4 per cent), six (4.2 per cent), and two (1.4 per cent) respectively. *L. ivanovii* and *L.grayi* were absent in screened samples.

Occurrence of Listeria contamination in an industrial poultry processing plant was investigated by Barbalho *et al*. (2005) by sampling carcasses at varying stages of processing and testing the hands and gloves of food handlers as well as the chilling water used in the process. They collected a total of 121 samples: 66 from carcasses, 37 from workers hands and gloves and 18 from the water used for chilling. Except for the water samples Listeria was isolated from all sampling sites. The species most often isolated was *L. innocua,* which accounted for 28 (90.3 per cent) isolates. Three (9.7 per cent) of the isolates were *L. monocytogenes* and two (6.5 per cent) were *L. grayi*. The frequency of Listeria in the chicken carcasses was similar at bleeding, defeathering and at end of evisceration stages (33.3 per cent), the level of *Listeria* spp. decreased during scalding (16.7 per cent), and increased immediately after initial evisceration stage (50 per cent) to peak after packaging (76.2 per cent). The carcasses were contaminated by *L. monocytogenes* serotypes 1b and 1c only during packaging. The prevalence of *Listeria* spp. on workers hands and gloves was 46 per cent mostly with *L. innocua* (40.5 per cent) followed by *L. monocytogenes* 1b (11.8 per cent).

Listeria spp. were present in 43 out of 70 samples of raw poultry meat screened by Kosek-Paszkowska *et al*. (2005) and six of Listeria strains were defined as *L. monocytogenes*. Fifty samples of heat-treated products of chicken examined were free of *Listeria* spp.

A study on the prevalence of *Listeria* species in meat products in Ankara, Turkey were carried out by Yucel *et al.* (2005). In this, a total of 146 raw and cooked meat samples were analysed for the presence of *Listeria* spp. Incidence of *Listeria* species in raw and cooked chicken meat were 69 per cent and 17.8 per cent respectively. *L. monocytogenes* was isolated from 11.5 per cent of raw chicken and 3.5 per cent of cooked chicken meat examined. Other species isolated included *L.innocua* 57.6 per cent from raw chicken samples and *L.innocua* 10.7 per cent and *L. murrayi* in 3.5 per cent of cooked chicken meat.

Incidence of *Listeria* spp. was assessed by Jalali and Abedi (2008) from 66 samples of fresh poultry and 4.5 per cent of samples were found positive for *L.innocua*. *Listeria* species could not detected in the screened six samples of frozen poultry.

Peckrovic *et al*. (2008) reported that *L. monocytogenes* was not detected by either conventional method or PCR technique using primers targeting the (*hly*A) gene from raw chicken samples in Croatia.

Filiousis *et al*. (2009) reported that the prevalence of *L. monocytogenes* in 20 samples of chicken meat from Greek open-air Markets as 35 per cent.

Malek *et al*. (2010) conducted a study on occurrence of Listeria in 25 samples each of frozen chicken legs and frozen chicken breast fillets collected from Assiut city, Egypt. *Listeria* spp. were detected in 13 frozen chicken leg and 14 frozen chicken fillet samples analyzed. PCR results confirmed *L.monocytogenes* in two of frozen chicken legs examined with the total incidence of five isolates from the 100 food samples examined.

Pesavento *et al.* (2010) conducted a research survey to check the presence of *Listeria* spp. in raw meat and retail products. Total prevalence was 11.7 per cent (148/1268) and in poultry meat was 21.6 per cent (45/208). Some samples contained two or three different strains of *Listeria* species. Among the 53 isolated strains of *Listeria* species in chicken *L.monocytogenes, L.innocua, L.welshimeri, L.ivanovii* and *L.grayi* were 13 (24.5 per cent), 21 (39.6 per cent), 11 (20.7 per cent), seven (13.2 per cent) and one (1.89 per cent) respectively. *L.seeligeri* was not detected from chicken.

Stonsaovapak and Boonyaratanakornkit (2010) screened a total of 380 meat and meat products, dairy and dairy products, fresh vegetables, fresh seafood, and ready-to-eat food samples collected from supermarkets in Bangkok, Thailand. The overall incidence of *Listeria* spp. was 16.8 per cent, most of them were isolated from raw meat and vegetables. *L. monocytogenes* was isolated from 18 (4.7 per cent) out of 380 studied samples. When analysed 30 samples of chicken liver, 12 (40 per cent) were positive for *Listeria* species and *L.monocytogenes* were detected only in four (13.3 per cent) samples.

Vasilev *et al*. (2010) surveyed ready-to-eat foods in Israel over a period of ten years, 1998–2007 for the presence of *L.monocytogenes*. The level of incidence of *L.monocytogenes* in poultry samples were 27 per cent among screened 1050 samples. The highest incidence was noticed in poultry samples.

Yan *et al*. (2010) collected a total of 2177 food samples from nine cities in northern China during 2005 to 2007 and were screened for the presence of *L. monocytogenes*. Contamination with *L. monocytogenes* was detected in 4.13 per cent of the total samples representing various food products. The isolation rate in raw meat products was 6.28 per cent where as in cooked meat was 3.31 per cent. Among raw meat samples, the pathogen was detected among 8.94 per cent of raw chicken meat.

Osaili *et al.* (2011) studied the prevalence of *Listeria* spp. in raw chicken and ready-to-eat chicken products in Amman, Jordan. A total of 280 raw chicken and ready to eat chicken products (chicken-shawirma, chicken-burger, chicken-sausage and mortadella) were collected from Amman abattoir and local retail markets in Amman city. *Listeria* spp. was isolated by the conventional International Organization for Standardization (ISO) method and *L. monocytogenes* was identified by biochemical and Polymerase Chain Reaction. Results of conventional method showed that out of 280 samples examined, 141 (50 per cent) were found to be contaminated with *Listeria* spp. [*L. monocytogenes* (18.2 per cent), *L. ivanovii* (26.1 per cent), *L. grayi* (3.5 per cent), *L. seeligeri* (1.8 per cent) and *Lwelshimeri* (0.7 per cent)]. The PCR confirmed all *L. monocytogenes* isolates (51 isolates: 15 from raw dressed broiler chicken, 23 from chicken-burger, nine from chicken sausage, and four from chicken-shawirma).

2.2.3. Seafoods

Fuchs and Surendran (1989) noted the absence of *L.monocytogenes* in fish and fish products from Cochin, India, although 35.7 per cent of the fresh fish samples harboured *Listeria* spp. None of the dried fish were positive for *Listeria* species.

Manoj *et al*. (1991) studied fish and fish handling areas around Manglore for the presence of *Listeria* species. None of the fish (12 samples) and shrimp (one sample) taken from fish landing centre were positive for *Listeria* species whereas one out of six samples of swabs taken from decks of fishing vessel, fish carrying baskets, floors of shrimp processing units and other shrimp contact surfaces in these units were positive for *L.innocua*. From fish market, two out of 39 samples of fish were positive for *Listeria* spp. and the isolated species were *L.seeligeri* and *L.grayi*, and none of the shrimp (two samples) were positive. One out of six samples of shrimp, one in ten samples of processed shrimp and three in nine swab samples were positive for *Listeria* spp. and the isolated species were *L.murrayi*, *L.innocua* and *L.grayi*. In total eight out of 95 samples were positive for *Listeria* spp.

A total of 234 samples of food, consisting of 158 raw and 76 samples of ready-to-eat food were examined by Arumugaswamy *et al*. (1994) for the presence of *L. monocytogenes*. The frequency of *L. monocytogenes* contamination in raw prawns was 44 per cent.

Based on a prevalence study conducted by Jemmi and Keuch (1994) *L. monocytogenes* was prevalent in 10 per cent of seafood samples screened in Switzerland.

Ng and Seah (1995) studied the incidence of *L.monocytogenes* from a range of foods in Singapore. They detected three out of 16 fish fingers/ fish cakes and one out of two of smoked mussels were positive for *L.monocytogenes*. None of the crab meat (0/16) and tuna fish (0/5) was positive for *L.monocytogenes*.

A smoked salmon processing plant including a smokehouse and a slaughterhouse was examined by Rorvik *et al.* (1995) for the occurrence of *L. monocytogenes* and other *Listeria* spp. From a total of 475 samples the overall frequency of *L. monocytogenes* was 16 per cent, while other *Listeria* spp. were found in 22 per cent of the samples. *L. monocytogenes* was most often detected in samples from the smokehouse, where 29 per cent of the environmental and 26 per cent of the fish samples contained the bacteria. Seventeen per cent of the fish raw material to the smokehouse was contaminated, while 11 per cent of the samples from vacuum-packed smoked salmon were positive for *L. monocytogenes.* The slaughterhouse was sporadically contaminated, but *L. monocytogenes* was not found in 50 samples of slaughtered fish. *L. monocytogenes* could be detected from seawater outside the slaughterhouse.

Bansal *et al*. (1996) tested 130 samples of seafoods by the PCR procedure. Food samples were collected from local food outlets by the NSW Health Department’s food inspectors, Australia. The seafoods selected include smoked salmon, smoked trout, crab highlighter, salmon products and others. In total 32 per cent samples were positive for *Listeria* species and out of these 20 per cent were confirmed as *L.monocytogenes*. *L.monocytogenes* were present in smoked salmon, smoked trout, crab highlighter, salmon products and others as ten, one, two, twelve, and one per cent respectively and *Listeria* species were present in fourteen, three, three, seventeen and four per cent of samples respectively.

The incidence of *Listeria* spp. in tropical fish and shellfish was studied by Jeyasekaran *et al.* (1996). Incidence of *Listeria* species in finfish and shellfish were 72.4 per cent and 44.4 per cent respectively. *L. monocytogenes* could be detected in 17.2 per cent of finfish and 12.1 per cent of shellfish. The incidence of *L.innocua* in finfish and shellfish were 65.5 per cent and 36.1 per cent respectively. *L.welshimeri* were absent in finfish and 5.6 per cent were there in shellfish. *L. innocua* was the most common species encountered. In 6.9 per cent finfish and 5.6 per cent shellfish, both *L. monocytogenes* and *L. innocua* were detected.

Forty smoked salmon processing plants were screened for the occurrence of *L. monocytogenes* and other *Listeria* spp. in the smoked salmon and in the drains by Rorvik *et al*. (1997). *L. monocytogenes* was detected from 33 per cent of smoked salmon and 63 per cent of drain samples from plants. Other *Listeria* spp. were found in 40 per cent of smoked salmon samples and 75 per cent of drain samples from the plants.

Johansson (1998) investigated on the occurrence of *L. monocytogenes* from foodstuffs and food-processing environments. Among 110 samples of vacuum packed smoked and cold salted fish screened, 22 were positive by the culture method.

Jorgensen and Huss (1998) studied on the prevalence of *L. monocytogenes* in naturally contaminated seafood. The highest prevalence was found in cold-smoked fish (34–60 per cent), while the lowest was found in heat-treated and cured seafood (4–12 per cent). The prevalence of *L. monocytogenes* differed greatly in cold-smoked salmon between production sites, ranging from 1.4 to 100 per cent.

Autio *et al*. (1999) assessed the sources of *L.monocytogenes* contamination in a cold-smoked rainbow trout processing plant. They reported that the frequency of raw fish samples containing *L. monocytogenes* was low and most contamination occurs during processing.

Duarte *et al*. (1999) tested fish samples collected along the processing chain of cold-smoked fish. *Listeria* spp. and *L. monocytogenes* were respectively present in 56 and 34 of 315 samples analysed.

Johansson *et al.* (1999) studied on the occurrence of *L. monocytogenes* strains in 110 samples of retail vacuum packed fish products in Finland. The incidence in hot smoked, cold smoked and cold salted were two per cent, 17 per cent and 50 per cent respectively.

A survey was performed in 1994 and 1995 in retail foods in Denmark by Norrung *et al.* (1999) for detecting level of *L. monocytogenes*. Incidence of *L.monocytogenes* in raw fish and preserved fish products (non heat treated) were 14.2 per cent and 10.8 per cent respectively.

Destro (2000) investigated on incidence of Listeria in fish and fish products from Latin America. Both *L.monocytogenes* and *L.innocua* were detected from three out of 63 samples of shrimp. Among 142 other fish products screened, incidence of *L.monocytogenes* and *L.innocua* were six (4.2 per cent) and four (2.8 per cent), respectively.

The situation in Canada regarding *L. monocytogenes* and ready-to-eat seafood products were investigated by Farber (2000). The incidence of *L. monocytogenes* in imported seafood products in 1996–1997 and 1997–1998 was 0.88 and 0.3 per cent respectively. With respect to domestic products, an analysis of 347 ready to eat foods in 1997–1998 and 1998–1999, at one of the large fish inspection labs in the Maritimes, revealed absence of *L. monocytogenes*.

Karunasagar and Karunasagar (2000) reviewed various research works on prevalence of *Listeria monocytogenes* in tropical fish and fish products and reported that the organism was comparatively low in tropical environments and hence the risk associated with the consumption of these products is also low.

Jeyasekaran *et al.* (2001) compared two selective media PALCAM and Listeria Selective Agar Medium Modified (LSAMM) for the isolation *of Listeria* spp. from seafoods. The incidence of *Listeria* species in raw seafoods, frozen seafoods, smoked seafoods, and salt dried seafoods were thirty seven out of sixty five, eight out of seventy two, ten out of twenty two and six out of twenty nine respectively. Out of the 61 isolates of *Listeria* species obtained from seafoods, 56 were recovered on PALCAM agar whereas LSAMM yielded only 29 isolates**.**

Jemmi *et al*. (2002) studied on the prevalence and risk factors for contamination with *L. monocytogenes* of imported and exported meat and fish products in Switzerland, 1992–2000. Incidence of *L. monocytogenes* in cold smoked fish (814), hot smoked fish (471), marinated fish (125), other ready to eat fish and seafood products (151) and raw fish (28) were 14 per cent, 12 per cent, 38 per cent, seven per cent and 11 per cent, respectively.

Dhanashree *et al*. (2003a) examined fresh, dry and smoked seafood, dairy products, fresh meat and vegetable samples to determine incidence of *Listeria* spp. in food samples collected from retail outlets in Manglore during the period March, 1997 to December, 2001. Among 210 samples of seafoods incidence of *L.monocytogenes* and *L.innocua* were 0.95 per cent and 22.9 per cent, respectively. The level of *L. monocytogenes* in fresh flat fish and clams were 2.9 per cent and 4.2 per cent and *L.innocua* were 37.1 per cent and 4.2 per cent, respectively. Dry flat fish and smoked tuna were negative for both *L.monocytogenes* and *L.innocua*. *L.innocua* detected from lactarius (50 per cent), mackerels (7.1 per cent), crabs (78.6 per cent), croakers (54.5 per cent), pomfret (16.7 per cent), fresh prawn (9.1 per cent), dry prawn (11.1 per cent) and sardines (33.3 per cent).

A total of 2522 samples, including processing lines and environment, personnel, raw materials and products (raw and ready-to-eat) were analysed by Gudbjornsdottir *et al*. (2004) for the presence of *L. monocytogenes* from six meat, five seafood and two poultry processing plants. The overall incidence of *L. monocytogenes* in seafood plants varied from 5.9 per cent to 22.1 per cent. Overall frequency of *Listeria* spp. and *L. monocytogenes* in 1180 seafood processing plant samples were 169 and 154, respectively.

Mena *et al*. (2004) screened 1035 different food products commercialized in Portugal. The incidence of *L. monocytogenes* in raw fish was 12 per cent (3/25). None of the shellfish sample out of eight sample examined was positive for *L.monocytogenes*.

L.monocytogenes contamination of ready-to-eat seafood products commercially available in Osaka was examined by Nakamura *et al.* (2004) between 1999 and 2000. *L.monocytogenes* was isolated from 12 out of the 95 products tested. All positive samples were from cold-smoked fish with nine being obtained during the summer.

In a study on occurrence of *L.monocytogenes* in fresh and processed foods in Navarra, Spain, Vitas and Garcia- Jalon (2004) screened 3685 samples. Out of 100 smoked salmon samples 41 samples contained one or more species of Listeria. *L.monocytogenes* was present in 28 samples. Other *Listeria* species were *L.innocua* (12 samples) and *L. seeligeri* (one sample).

Based on the prevalence study conducted by Busani *et al*. (2005) *L.monocytogenes* was prevalent in 2.4 per cent of 42,300 various food samples screened and contamination rate was 6.5 per cent in fish.

Gudmundsdottir *et al*. (2005) evaluated *Listeria* spp. and *L. monocytogenes* contamination of cold-smoked salmon (125 samples) and its processing environment (522 samples) during surveys conducted in 1997–1998 and 2001 as well as in samples of final products analysed in 2001. The overall frequency of *Listeria* spp. and *L. monocytogenes* in samples from all sources were 15.1 per cent and 11.3 per cent, respectively, but the incidence of *L. monocytogenes* in cold-smoked salmon final products was only four per cent.

Miettinen and Wirtanen (2005) investigated on prevalence of *L. monocytogenes* in farmed rainbow trout. The prevalence of *Listeria* spp. and *L. monocytogenes* in pooled unprocessed fresh rainbow trout was 35.0 per cent and 14.6 per cent respectively. On the other hand, the prevalence of *Listeria* spp. and *L. monocytogenes* in individual thawed fish was found to be 14.3 per cent and 8.8 per cent respectively. Up to 95.6 per cent of the *L. monocytogenes* and 84.5 per cent of *Listeria* spp. positive samples were gill samples. Only 4.4 per cent (2/45) of the *L. monocytogenes* positive samples were obtained from skin or viscera.

Hedge *et al*. (2007) screened 50 food samples collected from retail outlets using the USDA-FSIS method for the detection and isolation of *L.monocytogenes* utilizing BBL CHROM agar Listeria and Modified Oxford as selective agar. The food samples included vegetables, milk and milk products, meat products, seafood, poultry products, ready to eat products and mushroom. The seafoods selected include shrimp, mussels, lobster, crab, mackerel, herring, salmon, and seafood sauce. Only two samples were positive for *L.monocytogenes.*

An epidemiological survey of *L. monocytogenes* was done by Cruz *et al.* (2008) in a Gravlax salmon processing line. Four hundred and fifteen samples were collected at different steps of a gravlax salmon processing line in Sao Paulo state, Brazil. *L. monocytogenes* was confirmed in salmon samples (41 per cent), food contact surfaces (32 per cent), non- food contact surfaces (43 per cent) and of food handler’s samples (34 per cent), but could not be detected in any ingredient.

Incidence of *Listeria* spp. was assessed by Jalali and Abedi (2008) from 12 samples of shrimp and *L.innocua* was isolated from one sample. When screened 12 samples of frozen fish *Listeria* species could not be detected. From 61 samples of fresh fish, *L.monocytogene*s and *L.seeligeri* could not be detected where as *L.innocua* and other *Listeria* species one each was detected.

The incidence of *Listeria* species in seafood samples of markets in Goa was studied by Parihar *et al*. (2008). One hundred and fifteen raw/ fresh seafoods bought at the fish markets were sampled and tested for presence of *Listeria* spp. Twenty eight seafood samples were positive for *Listeria* spp. and in ten samples *L. monocytogenes* was detected. *L.innocua* was the most common *Listeria* species recovered and was detected in 18 samples.

The prevalence of *L.monocytogenes* in ready-to-eat products of markets in Northern Spain was studied by Garrido *et al.* (2009). The samples analysed were deli meat products, smoked fish and pate (783). Ready to eat smoked fish was the most frequently contaminated food category (25 per cent positive), with high occurrence in some brands (60 per cent of lots positive). They analysed 102 samples of vacuum packed smoked salmon and 19 samples were positive for *Listeria* species and 11 isolates were confirmed as *L.monocytogenes*. The other *Listeria* species isolated were *L.welshimeri* (7.8 per cent) and *L.innocua* (1 per cent). From 40 samples of vacuum packed smoked trout, 14 samples were positive for multiple contaminations of *Listeria* species. Ten isolates were *L.monocytogenes* and five were confirmed as *L.innocua.*

A work was undertaken by Gianfranceschi *et al*. (2009) to study the serotypes of 674 *L.monocytogenes* isolates collected from different Italian geographical areas during 2002–2005 and among that 558 were from foods. The different serotypes obtained from 17 isolates of raw fish were 1/2a (11), 1/2b (3), 3b (1), 4b (1), and nt (1). The serotypes of *L.monocytogenes* in 55 isolates from ready to eat fish were 1/2a (34), 1/2b (6), 3a (5) and 4b (10).

Uyttendaele *et al*. (2009) studied on prevalence of *L. monocytogenes* in ready to eat food (1974 samples). *L. monocytogenes* was detected in 27.8 per cent (25/90) of smoked fish samples.

Prevalence and contamination patterns of *L. monocytogenes* in catfish processing environment and fresh fillets were evaluated by Chen *et al.* (2010). Catfish skin, intestines, fresh fillets, processing surfaces at different production stages, chiller water and non-food contact surfaces were sampled for *L. monocytogenes* and other *Listeria* species. Among 315 samples, prevalence of *L. monocytogenes*, *L.innocua* and a group of *L. seeligeri*- *L.welshimeri*- *L.ivanovii* was 21.6, 13.0 and 29.5 per cent respectively. No *L. grayi* was detected in this survey. While no *L. monocytogenes* strains were isolated from catfish skin and intestines, the strains were found with a frequency of 76.7 per cent in chilled fresh catfish fillets and 43.3 per cent in unchilled fillets. *L. monocytogenes* and *Listeria* spp. were also detected in fish contact surfaces such as deheading machine, trimming board, chiller water, conveyor belts at different stages, and fillet weighing table. Among *L. monocytogenes*, 1/2b (47 per cent), 3b (16 per cent) and 4c (14 per cent) were the predominant serotypes isolated, whereas 4b, 4e, 1/2c and 1/2a were detected at much lower frequencies.

Pesavento *et al*. (2010) conducted a research survey to check the presence of *Listeria* spp. in raw meat and retail products. Overall prevalence was 11.7 per cent (148/1268) and no sample was found positive among 19 smoked salmon samples screened.

Pinto *et al.* (2010) studied on the prevalence of *L. monocytogenes* in ready-to-eat foods from supermarkets in Southern Italy. The pathogen was detected in 105/1045 ready to eat food samples. Forty five out of 132 (34.1 per cent) smoked salmon samples were positive for *L.monocytogenes.*

Stonsaovapak and Boonyaratanakornkit (2010) screened a total of 380 meat and meat products, dairy and dairy products, fresh vegetables, fresh seafood, and ready-to-eat food samples from supermarkets in Bangkok, Thailand. The overall incidence of *Listeria* spp. was 16.8 per cent, most of them were isolated from raw meat and vegetables. *L. monocytogenes* was isolated from 18 out of 380 studied samples. When analysed 20 samples each of shrimp, fish and squids, none where positive for *L.monocytogenes*. *Listeria* species were present in both shrimp (ten per cent) and squids (five per cent). They were not able to isolate any *Listeria* species from fish.

Vasilev *et al.* (2010) surveyed *L. monocytogenes* strains, isolated from ready-to-eat foods in Israel over a period of 10 years, 1998–2007. The level of incidence of *L.monocytogenes* in poultry samples was 15 per cent among screened 3063 samples.

Yan *et al.* (2010) collected a total of 2177 food samples from nine cities in northern China during 2005 to 2007 and were screened for the presence of *L. monocytogenes*. Contamination with *L. monocytogenes* was detected in 4.13 per cent (90/2177) of the total samples representing various food products. The isolation rate in seafoods was 1.17 per cent (4/343).

The occurrence of *Listeria* spp. and *L. monocytogenes* in retail RTE meat and fish products in Vancouver, British Columbia (B.C.) was investigated by Kovacevic *et al.* (2012). Conventional methods were used to recover *Listeria* spp. from deli meat and fish samples 40 each collected from 17 stores. Fish samples consisted of different flavoured, candied and/ or smoked fish jerky, nuggets, and pepperoni samples, as well as lox, sockeye sticks, smoked steelhead trout, and tuna. *Listeria* spp. were recovered only from fish samples (20 per cent); five per cent harboured *L.innocua*, five per cent had *L. monocytogenes* and ten per cent contained *L.welshimeri*.

2.3. ANTIBIOTIC SUSCEPTIBILITY

The susceptibility of 148 strains of *L.* monocytogenes isolated from foods to antibiotics currently used in veterinary and human therapy was determined by

Aureli *et al*. (2003) using standard agar dilution and disk diffusion methods. The antibiotics included amikacin, amoxycillin, cefazolin, chloramphenicol, erythromycin, flumequine, fosfomycin, gentamicin, kanamycin, lincomycin, oxytetracycline, rifampicin, spiramycin, streptomycin, tetracycline, tobramycin and vancomycin. All the strains displayed sensitivity to the tested antibiotics with the exception of fosfomycin, lincomycin and flumequine for which they were all resistant. The strains were moderately sensitive to only four antibiotics: spiramycin, chloramphenicol, tetracycline and streptomycin.

Kiss *et al.* (2006) monitored food samples for contamination with *L. monocytogenes* and the incidence of human listeriosis in Hungary in the year 2004. On the basis of the antibiotic sensitivity test results of strains isolated from the disease cases, penicillin and aminoglycoside antibiotics or a combination of these were found to be effective.

Conter *et al.* (2009) evaluated the susceptibility of 120 *L. monocytogenes* strains isolated from food and food-processing environments to 19 antibiotics currently used in veterinary and human therapy. The antibiotics used were penicillin, ampicillin, ampicillin/ sulbactam, oxacillin, imipenem, gentamicin, ciprofloxacin, moxifloxacin, erythromycin, clindamycin, quinopristin/ dalfopristin, linezolid, teicoplanin, vancomycin, tetracycline, fosfomycin, fusidic acid, rifampicin and trimethoprim/ sulfamethoxazole (TMP- SMX). Among the 120 tested strains, 14 (11.7 per cent) displayed resistance to at least one antibiotic. 8.3 per cent isolates were resistant to one antibiotic, 2.5 per cent to two antibiotics and 0.8 per cent to five antibiotics. Resistance to clindamycin was the most common with four isolates showing resistance and six strains showing an intermediate behaviour to this antibiotic. Linezolid was the next most common, with four resistant isolates, then ciprofloxacin (two each of resistant and intermediate), ampicillin and rifampicin (two), TMP–SMX (two) and, finally, vancomycin and tetracycline (one each).

Pesavento *et al. (*2010) conducted a survey to check the presence of *Listeria* spp. in raw meat and retail products and to analyse their antibiotic resistance. The susceptibility of 168 strains of *Listeria* spp. was determined by disk diffusion method: They found out that 8.33 per cent of isolates were susceptible for all antibiotics used. All isolated strains, except one, were sensitive to one of the first choice antibiotics (ampicillin and gentamicin) or to trimethoprim–sulfamethoxazole used as antibiotic of second choice in the treatment of human listeriosis. Strains isolated from ready-to-eat food showed high level of resistance to ampicillin, gentamicin and meticillin. They reported that the level of single resistance was higher in *L. monocytogenes, L.innocua and L. seeligeri* than in *L.ivanovii, L.welshimeri* and *L. grayi* that showed higher percentage of multi resistance.

Stonsaovapak and Boonyaratanakornkit (2010) screened a total of 380 meat and meat products, dairy and dairy products, fresh vegetables, fresh seafood, and ready-to-eat food samples from supermarkets in Bangkok, Thailand. The antimicrobial susceptibility of the 64 isolates of *Listeria* spp. was examined by the standard disk diffusion method. Resistance to penicillin was most evident, with four isolates showing resistance to this antibiotic. Chloramphenicol was the next most evident, with two isolates showing resistance, then tetracycline with one isolate. All *Listeria* isolates were sensitive or displayed intermediate sensitivity to ampicillin, amoxycillin and vancomycin. The level of resistance in *L. monocytogenes* was low (5.6 per cent) compared with *L.innocua* (16 per cent), *L.ivanovii* (33.3 per cent) and *L. seeligeri* (50 per cent). No resistance was observed with *L. grayi* or *L.welshimeri*. Resistance to one antibiotic was more common than multiple resistances; only *L.innocua* showed resistance to multiple antibiotics.

Yan *et al*. (2010) collected a total of 2177 food samples from nine cities in northern China during 2005 to 2007 and were screened for the presence of *L.monocytogenes*. Contamination with *L. monocytogenes* was detected in 4.13 per cent (90/2177) of the total samples representing various food products.

Antimicrobial resistance was most frequently observed for ciprofloxacin (17.8 per cent), tetracycline (15.6 per cent) and streptomycin (12.2 per cent). Overall, resistance was observed against 14 out of 18 antimicrobials tested while multiple resistances occurred among 18.9 per cent (17/90) isolates. Two isolates were resistant to more than five antimicrobials.

Antibiotics sensitivity test was assayed on ten isolates of Listeria from retail meat (beef) tables in Ibadan municipal abattoir, Nigeria by Adetunji and Isola (2011). The isolates were tested for sensitivity for eight commonly used antibiotics using Bauer-Kirby disc diffusion assay. Antibiotics sensitivity profile showed that all the isolates were resistant to three or more antibiotics. The overall incidence of antibiotics resistance in Listeria strains was relatively lower (37.5 per cent). In the study, *L. monocytogenes* and *Listeria* spp. showed resistant to three out of the eight (38.5%) antibiotics used. The least sensitive drugs for *L.monocytogenes* and other *Listeria* spp. were cloxacillin, tetracycline and chloramphenicol. *L. monocytogenes* was highly sensitive to gentamicin and streptomycin. *Listeria* spp. were highly sensitive to gentamicin, erythromycin and streptomycin.

Osaili *et al.* (2011) determined the prevalence of *Listeria* spp. and antibiotic susceptibility of *L. monocytogenes* in raw chicken and ready-to-eat (RTE) chicken products in Amman, Jordan. A total of 280 raw chicken and RTE chicken products were collected from Amman abattoir and local retail markets in Amman city. Incidence of *L. monocytogenes* was 18.2 per cent (51 isolates). Ten antibiotics used in the study were erythromycin, gentamicin, tilimicosin, enrofloxacin, ciprofloxacin, doxycycline, tetracycline, amoxicillin, trimethoprim, and florfenicol. Five of the tested *L. monocytogenes* isolates were resistance to two antibiotics (tilimicosin and tetracycline).

Bouayad and Hamdi (2012) obtained six isolates of *L. monocytogenes* from 227 food samples. The following antibiotics were used to determine the antibiotic sensitivity: gentamicin (10 mg), piperacillin (30 mg), tetracycline (30 mg) (BIORAD), trimethoprim/ sulfamethoxazole (1.25/23.75 mg), amoxycilin/ clavulanic acid (20/10 mg) and chloramphenicol (30 mg) (OXOID). All the *L. monocytogenes* strains showed sensitivity to all antibiotics tested.

The occurrence of *Listeria* spp. and *L. monocytogenes* in retail RTE meat and fish products in Vancouver, British Columbia (B.C.) was investigated by Kovacevic *et al.* (2012). Antimicrobial resistance (AMR) profiling showed that all *Listeria* spp. possessed resistance to cefoxitin and nalidixic acid. All *L.monocytogenes* isolates were resistant to cefoxitin, ciprofloxacin, nalidixic acid and clindamycin. Reduced susceptibility to amikacin and chloramphenicol was also observed in one *L. monocytogenes* and three *L.welshimeri* isolates, respectively.

Materials and Methods

3. MATERIALS AND METHODS

A study was undertaken to determine the prevalence and distribution of *Listeria monocytogenes* and other *Listeria* spp. in various food samples. A total of 385 food samples consisting of beef, chicken, prawns and sardines were randomly selected and purchased from retail outlets, local vendors and corporation slaughter house at Kuriachira in Thrissur for a period of nine months from July, 2011 to March, 2012. *Listeria* spp. are widely distributed in environment and all foods of animal origin including meat, milk, egg and fish can act as source of the organism. All the samples were subjected to isolation and identification of *Listeria* spp. by conventional culture techniques. The isolates of *Listeria* spp. obtained were subjected to confirmation by immunological and molecular methods. Details of the study and the experimental methods adopted are described in this section.

3.1. BIOLOGICALS, CHEMICALS AND REAGENTS

3.1.1 Bacterial Strains

Standard cultures of *L. monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), and *Rhodococcus equi* (MTCC 1135) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The isolates were maintained in Brain Heart Infusion (BHI) broth with ten per cent glycerol by sub culturing at regular intervals and periodically tested for their purity, morphological and biochemical characteristics.

3.1.2. Chemicals and Reagents

All the chemicals and reagents used in the study were of analytical grade. The composition of various buffers and reagents used in the study are appended in Annexure I. Microbiological media used for the isolation of the organism and various reagents for biochemical tests were procured from HiMedia, India. The composition of the media and specific procedure followed in its preparation are appended in Annexure II. Molecular reagents were procured from Banglore GeNei, India Limited and from Fermentas, Banglore.

3.2. COLLECTION OF SAMPLES

3.2.1. Beef Samples

One hundred and five fresh beef samples of 100g each were collected from retail outlets in Mannuthy and Thrissur, local vendors, and slaughter house at Kuriachira, Thrissur. The samples were taken from rump and back region as these regions were found to be contaminated more through contact with intestinal contents and hide.

3.2.2. Chicken Samples

A total of 150 samples of raw broiler chicken were collected from various retail outlets in Mannuthy and Thrissur. The samples were taken from birds in good condition and slaughtered by halal method. Hundred gram of sample was taken from each deskinned bird and thigh, rib, and neck area were selected for sampling.

3.2.3. Seafood Samples

3.2.3.1. Prawns

Fresh prawns of marine origin were collected from retail outlets in Mannuthy and Thrissur and from local vendors. A total of hundred samples (100g each) were collected for the study.

3.2.3.2. Sardines

Thirty samples of sardines (*Sardinella longiceps*) of 100 g each were collected from local vendors and retail outlets in Mannuthy and Thrissur. The local names of sardines are ‘Chaala’ and ‘Mathi’. It is the most preferred fish by common people and is the major source of omega three fatty acids. Samples were selected randomly.

3.3. TRANSPORTATION OF SAMPLES

The samples were collected aseptically in sterilized polythene bags and transported to the laboratory under chilled condition in thermocool containers. The samples were processed upon arrival in the laboratory and subjected to microbiological analysis on the same day of collection.

3.4. PROCESSING OF SAMPLES

The samples were aseptically and carefully freed from its casings. Then the samples were cut into small pieces and minced in a sterile stainless steel waring blender for three minutes. From the minced sample 25g was weighed and this formed the initial test sample.

3.5. ISOLATION AND IDENTIFICATION OF *LISTERIA* SPECIES

For the isolation and identification of *Listeria* spp. from various food samples, the U.S. Department of Agriculture (USDA) method described by Ryser and Donnelly (2001) was used with modifications.

Isolation of *Listeria* species from food was carried out by two step enrichment followed by selective plating in Polymyxin- Acriflavin- Lithium Chloride- Ceftazidime- Aesculin- Mannitol (PALCAM) (HiMedia) agar as described by Parihar *et al*., 2008 (Fig. 1).

3.5.1 Primary Selective Enrichment

Enrichment procedures were used to isolate low numbers of *Listeria* species from meat and seafoods. For the primary selective enrichment University of Vermont Medium (UVM) I (HiMedia) was used. Acriflavin (six milligram)

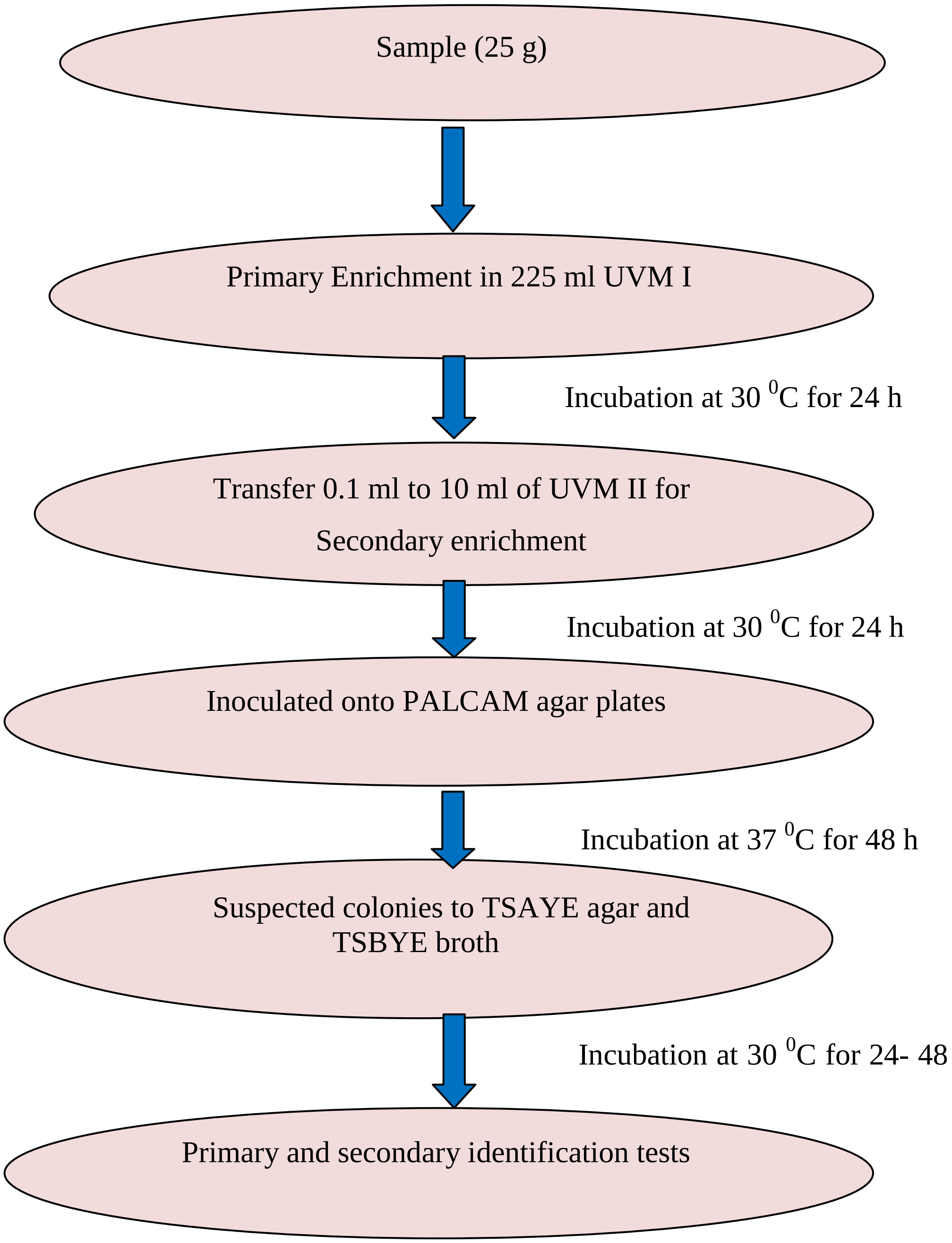

Fig. 1 Isolation of *Listeria* species

and Nalidixic acid (ten milligram) are the selective agents used in the UVM I media. Twenty five grams of each sample was added to a stomacher bag containing 225ml of UVM I. The mixture was homogenized using a laboratory blender for two minutes and incubated at 30 ^{0}C for 24 h.

3.5.2. Secondary Selective Enrichment

The secondary selective enrichment was carried out in University of Vermont Medium II (UVM II) (HiMedia) which contain the selective agents at higher concentration (Acriflavin 12.5 mg and Nalidixic acid 10 mg). From the primary enriched UVM I broth culture, 0.1 ml was transferred to 10 ml of UVM II broth and incubated at 30 ^{0}C for 24 h.

3.5.3. Selective Plating

After the period of incubation in UVM II broth, a loopful of the inoculum was streaked onto PALCAM agar plates. The plates were incubated at 37 ^{0}C for 48 h. On PALCAM Agar, colonies of Listeria appear gray-green with a black sunken centre and a halo. Identification of *Listeria* spp. on PALCAM agar plates was based on aesculin hydrolysis and mannitol fermentation.

For confirmation, five or more suspected colonies from PALCAM agar plates were transferred on to Trypticase Soy Yeast Extract Agar (TSYEA) (HiMedia) plates and incubated at 30 ^{0}C for 24 to 48 h. The selection of five colonies insured that multiple species of Listeria, if present, will be identified. The suspected individual colonies were then transferred to Trypticase Soy Yeast Extract Broth (TSYEB) (HiMedia) and incubated at 37 ^{0}C for 24 h. The isolates were then subjected to a series of biochemical tests for identification.

3.6. CHARACTERIZATION AND IDENTIFICATION OF ISOLATES

The suspected colonies of Listeria spp.were subjected to various tests and identified based on the cultural, morphological and biochemical characteristics described by Barrow and Feltham (1993) (Fig. 2). Schematic of biochemical identification for Listeria spp. are represented in figure 3.

Identification test	**Characteristics/ reaction**
Gram's reaction and cell morphology	**Gram positive short rods**
Catalase	**+**
Oxidase	**-**
Oxidation Fermentation	**Fermentative**
Motility	**Motile at 20-25 ^{0}C**
Methyl Red	**+**
Voges Proskauer	**+**
Citrate utilization	**-**
Urease activity	**-**
Phophatase	**+**
Aesculin hydrolysis	**+**
Indole production	**-**
Gelatin hydrolysis/ liquefaction	**-**
Hippurate hydrolysis	**+**
Carbohydrate utilization test and pathogenicity assay	

Fig. 2 Identification tests for *Listeria* species

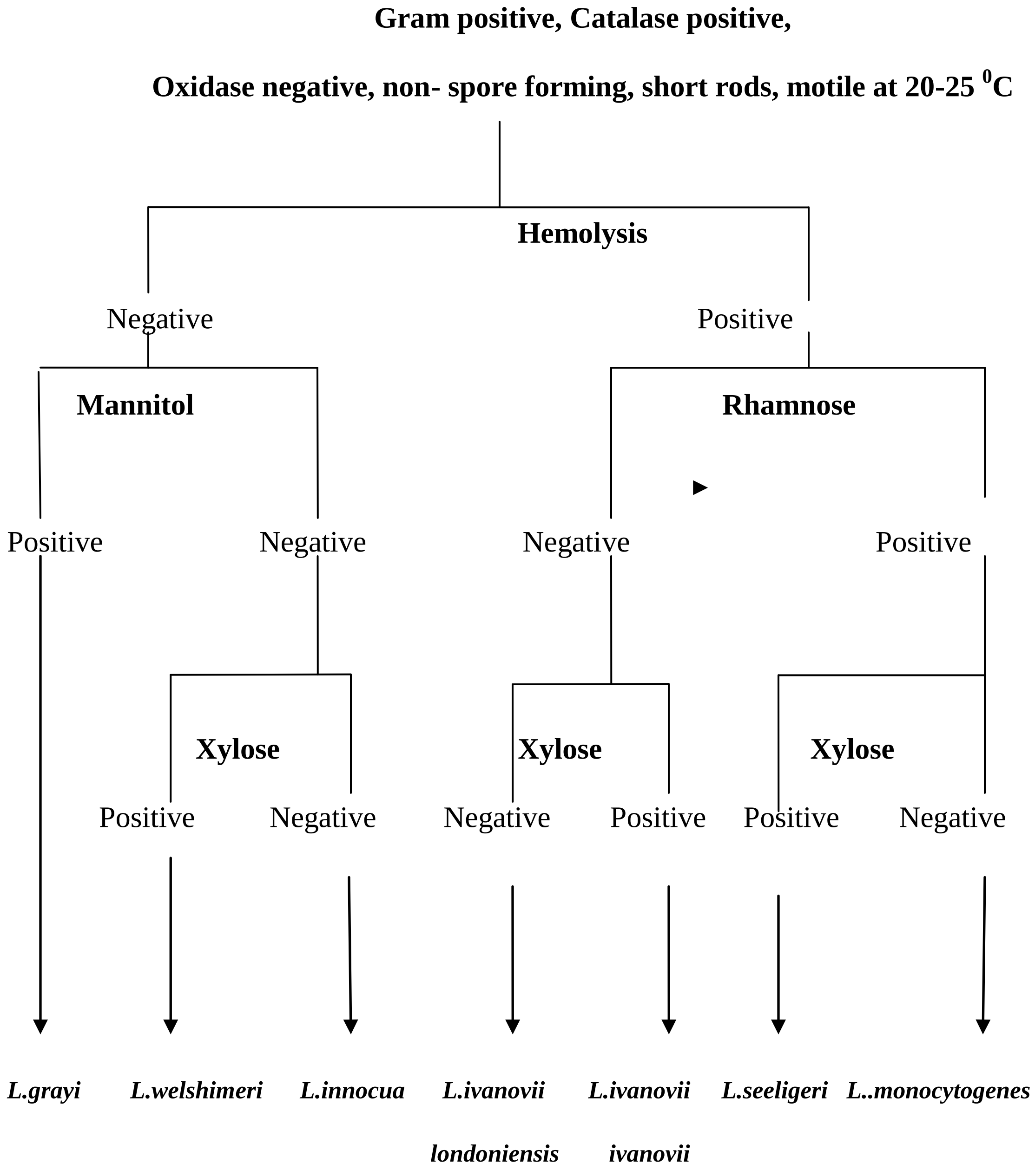

Fig. 3 **Schematic of biochemical identification for *Listeria* species**

3.6.1 Primary Identification Tests

3.6.1.1 Gram Staining

A thin smear of each isolate was made on a clean, grease free glass slide. The smear was air dried and then heat fixed by passing over a flame. The smear was then flooded with 0.5 per cent crystal violet in water and allowed to act for one minute. The stain was poured off and the smear washed with tap water. Then it was flooded with Gram's iodine solution (one per cent iodine and two per cent potassium iodide in water) for 30 sec. The solution was poured off and the smear was decolourised with a few drops of acetone for about 30 sec by gently agitating the slide till no colour come out of the smear. Smear was washed and counter stained with dilute carbol fuchsin for 30 sec. The stain was poured off from the slide, washed with tap water, air dried and examined under oil immersion objective of the microscope. The *Listeria* spp. appeared as gram positive short rods or cocco bacilli.

3.6.1.2. Catalase Test

i) Slide test

A small quantity of the inoculum was transferred onto a clear, grease free, glass slide and mixed well with a drop of three per cent hydrogen peroxide. Appearance of effervescence within a few seconds indicated a positive reaction.

ii) Tube test

Two to three millilitre of three per cent hydrogen peroxide solution was taken in a small test tube. Several colonies were taken using a sterile glass rod and immersed in the hydrogen peroxide solution. Active bubbling immediately indicated a positive reaction. Listeria colonies were catalase positive.

3.6.1.3. Oxidase Test

Oxidase discs (HiMedia) were used for testing oxidase producing capacity of the test organism. Using a sterile platinum rod removed a colony of the organism and smeared it on the disc. The appearance of a dark purple colour within 30 sec. indicated a positive reaction. Listeria organisms lack the capacity of producing oxidase.

3.6.1.4. Oxidation- Fermentation Test

Each isolate was inoculated into duplicate tubes of Hugh- Leifson medium (HiMedia) by stabbing with a straight wire. One of the tubes was sealed with a layer of melted soft paraffin to a depth of about three centimeter above the medium. The tubes were incubated at 37^0C for up to 14 days. A change in colour of the medium from green to yellow in the open tubes alone was taken as oxidation, whereas, a change in colour from green to yellow in both the tubes was regarded as fermentation. Absence of colour change in both tubes indicated no action on carbohydrates. *Listeria* spp. are fermentative in nature.

3.6.1.5. Motility Test

i) Tumbling motility test:

A wet mount was prepared from Brain Heart Infusion (BHI) broth (HiMedia) after 16 to18 h of incubation at 20 to 25°C. The wet-mount culture was examined under oil immersion objective of the microscope for small rods that exhibit an active end-over-end tumbling/ rotating movement which are characteristic of *Listeria* spp.

ii) Umbrella motility test:

Motility of the organism was assessed by stabbing the isolate into Listeria Motility Medium (HiMedia) with a straight wire up to a depth of 5mm and incubated at $37\ ^0$C for 24 h. Motility was indicated by a spreading growth into the medium from the line of inoculation and growth of non motile organism is confined to the stab. Umbrella like growth was shown by *Listeria* spp.

3.6.2. Secondary Identification Tests

3.6.2.1. Methyl Red (MR) Reaction

A small amount of the inoculum was transferred into five millilitre of MR-VP medium (HiMedia) in a tube and incubated at 37^0 C for two days. Two drops of methyl red solution was added at the end of incubation period and examined for colour reaction. Development of a red colour indicated positive reaction. *Listeria* spp. are MR positive.

3.6.2.2 .Voges Proskauer (VP) Reaction

A small amount of the inoculum was transferred into five millilitre of MR-VP medium in a tube and incubated at 37^0 C for two days. After incubation period, 0.6 ml of five per cent alpha naphthol solution and 0.2 ml of 40 per cent aqueous potassium hydroxide was added into the tube. The contents were mixed thoroughly and the tube was kept in a slanting position and examined after 15 min. and one hour. A positive reaction is indicated by the development of a strong red colour. *Listeria* spp. are VP positive.

3.6.2.3. Citrate Utilization Test

A light suspension of the organism was made in normal saline and was inoculated with a straight wire onto the slope of Simmon’s citrate agar (HiMedia). The inoculated medium was incubated at 37 ^{0}C and examined daily up to seven days. The ability of the organism to utilize citrate as the sole source of carbon was indicated by a change in colour of the medium from green to blue and growth of the organism along the streak line. *Listeria* spp. showed negative reaction to citrate utilization test.

3.6.2.4. Urease Activity

Slopes of Urea Agar Base (Christensen) (HiMedia) was heavily inoculated with the test organism and incubated at 37 ^{0}C. The tubes were examined after four hour of incubation and daily for five days. Development of a red colour in the medium indicated a positive reaction. Listeria organisms are urease negative.

3.6.2.5. Phosphatase Test

The Phenolphthalene Phosphate Agar (HiMedia) was lightly inoculated with the test organism to obtain discrete colonies and incubated at 37 ^{0}C for 18 h. At the end of the incubation, 0.1 ml of ammonia solution (specific gravity- 0.880) was placed in the lid of the Petri dish and the medium was inverted above it. Free phenolphthalene liberated by phosphatase react with the ammonia and phosphatase positive colonies became bright pink. *Listeria* spp. showed a positive reaction to phosphatase test.

3.6.2.6. Aesculin Hydrolysis

The organism was inoculated into aesculin broth and was incubated at 37^{0}C and examined daily for five days. Blackening of the broth due to hydrolysis of aesculin indicated a positive reaction. All *Listeria* spp. hydrolyse aesculin using aesculinase.

3.6.2.7. Indole Production

The isolate was inoculated into Tryptone Water (HiMedia) and incubated at 37^{0}C for 48 h. At the end of the incubation, 0.5 ml of Kovac’s reagent was added and mixed well and examined. A red colour in the reagent layer indicated a positive reaction. *Listeria* spp. are negative to indole production.

3.6.2.8. Gelatin Hydrolysis/ Liquefactions

Each isolate was inoculated into Nutrient Gelatin (HiMedia) and incubated at 37^{0}C up to 14 days. An uninoculated control tube was also set. The tubes were cooled every two to three days in a refrigerator for two hour and then examined for liquefaction. A positive result is indicated by liquefaction of gelatin. *Listeria* spp. do not hydrolyse gelatin.

3.6.2.9. Hippurate Hydrolysis

The slope of the hippurate agar was lightly inoculated with the test organism and examined daily for seven days. Hydrolysis of hippurate was indicated by growth and the development of a pink colour due to alkali production. *Listeria* spp. show a positive reaction to hydrolysis of hippurate.

3.6.2.10. Carbohydrate Utilization Test

A single carbohydrate disc was added to each tube containing Andrade Peptone Water (HiMedia) with an inverted Durham's tube and inoculated with test organisms. The inoculated tubes were incubated at 37 ^{0}C and examined daily for seven days to detect the production of acid and/or gas. A change in colour of the medium to pink indicated acid production and the production of gas was indicated by the appearance of air bubbles in the inverted Durham's tube. Anaerobic condition of the medium was provided by adding a layer of sterile molten soft paraffin to a depth of about one centimeter above the media. Carbohydrate utilization pattern of *Listeria* spp. is shown in table 1.

3.7. PATHOGENICITY TESTS

The pathogenic potential of Listeria isolates can be assessed by in vitro pathogenicity tests such as CAMP test and beta hemolytic activity. All the isolates were subjected to the pathogenicity tests. The details of the pathogenicity studies are given in the table 2.

3.7.1. Christie Atkins Munch Petersen (CAMP) test

The *Staphylococcus aureus* and *Rhodococcus equi* cultures were streaked in single lines on the Blood agar (HiMedia) plate. The cultures should be parallel and on opposite side of the agar plate. Thin inoculums were used.

Table 1. Carbohydrate utilization pattern of *Listeria* species

Sl. No.	**Acid from carbohydrate**	***L.monocytogenes***	***L.ivanovii***	***L.innocua***	***L.welshimeri***	***L.Seeligeri***	***L.grayi***
1	**D-mannitol**	-	-	-	-	-	+
2	**L-rhamnose**	+	-	**d**	**d**	**d**	-
3	**D-xylose**	-	+	-	+	-	-
4	**Dextrose**	+	+	+	+	+	+

d – 16 to 84 per cent strain positive

Table 2. Pathogenicity Assay of *Listeria* species

Item	**L. monocytogenes**	**L. ivanovii**	**L. innocua**	**L. welshimeri**	**L. seeligeri**	**L. grayi**
β hemolysis	+	++	-	-	w	-
CAMP test with S.aureus	+	-	-	-	+	-
CAMP test with R.equi	-	+	-	-	-	-

w - weak reaction

The test strain (as a thin line) was streaked at right angles to these cultures two millimeter apart from the *S.aureus* and *R.equi* lines. The plates were incubated at 37 ^{0}C for 18 to 24 h. Enhanced/ enlarged zone of hemolysis at the intersection of test strain with *S. aureus* and *R.equi* were noted. Enlarged zone of hemolysis with *S. aureus* confirmed *L.monocytogenes* where as a narrow zone indicated *L.seeligeri*. Enlarged zone of hemolysis with *R.equi* interpreted as *L.ivanovii*.

3.7.2. β Hemolysis

The isolated strains were streaked on blood agar plate and then it was incubated at 37 ^{0}C for 24-48 h. Hemolysis around the colony was noted in *L.monocytogenes, L. ivanovii* and *L. seeligeri*. The cytotoxic properties of hemolytic protein of these species caused the lysis of red blood cells on the agar plate. The hemolysis produced by *L. seeligeri* strains is weaker than by both *L. monocytogenes* and *L. ivanovii*.

3.8. IMMUNOLOGICAL METHOD

Lateral flow technique was used for confirmation of the *L.monocytogenes*. Singlepath L ‘mono (Merck), a Gold Labelled Immuno Sorbent Assay (GLISA) is a rapid test for qualitative detection and confirmation of *L.monocytogenes* in food and environmental samples. It is an immunological screening test and an extremely fast confirmation test for the specific detection of *L.monocytogenes* based on the immune flow principle.

Singlepath L ‘mono test device has a circular sample port, and an oval shaped test (T) and control (C) window. The sample applied on the chromatography paper via the circular port was absorbed through the pad to the reaction zone containing colloidal, gold labeled antibodies specific to *L.monocytogenes* antigen. The antigen makes complexes with the gold labeled antibody and migrates through the port until it encounters a binding zone in the test (T) area. The binding zone contained another anti- *Listeria monocytogenes* antibody, which immobilizes any *L.monocytogenes* antibody complex present. Due to the gold labeling a distinct red line is then formed. The rest of the sample continued to migrate to a second binding reagent zone within the control (C) zone, and also forms a second distinct line (positive control). Depending on the serotype, approximately 5×10^{6} bacteria/ millilitre is the lower detection limit.

All the isolates of *Listeria* spp. obtained in the study were subjected to immunological method using the principle of Lateral Flow Assay for confirming *L.monocytogenes*.

3.8.1. Sample Confirmation Assay

One to three suspected colonies from PALCAM media were resuspended in 250 microlitre Brain Heart infusion (BHI) broth (HiMedia) and incubated at 37^{0}C for one hour. It was allowed to cool to room temperature.

3.8.2. Test Procedure

A micro pipette and a pipette tip was used to deposit 150µl of BHI broth on the circular sample port of the Singlepath L ‘mono device. The test result was read within 30 min. The positive samples were differentiated by appearance of red line on both test (T) and control(C) zones and in negative samples only on control.

3.9. POLYMERASE CHAIN REACTION (PCR)

Conventional methods used for the detection of Listeria in foodstuffs are generally cumbersome and time consuming, requiring a minimum of five days to recognize *Listeria* spp. and about 10 days to identify *L. monocytogenes* by confirmatory tests. PCR is one of the most promising techniques for rapid detection of microorganisms in food. This process has provided increased sensitivity for detection and therefore enhanced the likelihood of detecting bacterial pathogens.

The Listeria isolates obtained from various foods were subjected to PCR as per the procedure described by (Gawade *et al.,* 2010) with slight modifications.

3.9.1. PCR Reagents and Chemicals

The reagents and chemicals used for the polymerase chain reaction were PCR reaction buffer (10X), Taq DNA polymerase, dNTP mix, $MgCl_2$, forward and reverse primer set and sterilized milliQ water. The materials needed for submarine agarose gel electrophoresis were Tris Boric acid EDTA (TBE) Electrophoresis buffer (10X), agarose gel, gel loading buffer and ethidium bromide.

3.9.2. Oligonucleotide Primers

The Oligonucleotide primers targeting the listeriolysin O gene (*hly* A) were used in the study for detection of *L.monocytogenes.* The primers were procured from Sigma–Aldrich, St. Louis, MO. The sequence of primers used in the study is listed in the table 3.

Table 3. Primers used for the identification of *L.monocytogenes (hly A* gene)

Primer	Length	Primer sequence	Amplification product size
Primer F	24	5′- GCA GTT GCA AGC GCT TGG AGT GAA -3′	460 bp
Primer R	24	5′-GCA ACG TAT CCT CCA GAG TGA TCG- 3′	460 bp

3.9.3. Reconstitution and Dilution of Primers

Primers obtained in lyophilized form were reconstituted in 100 microlitre of sterile triple distilled water to a concentration of 200 pico moles. The tubes were kept at room temperature with occasional shaking for one hour. They were spun briefly to pellet down the insoluble particles if any and the stock solution was redistributed into 10 µl aliquots and stored at -7 ^{0}C. At the time of use the aliquots were thawed and further diluted ten fold to obtain a concentration of 20 pico moles/ microlitre before using for PCR.

3.9.4. Preparation of DNA Template

For each Listeria strain, a loopful of cells grown overnight in BHI plates were transferred to BHI broth and incubated at 37 ^{0}C for 24 h. One millilitre of overnight culture of isolates were taken in an eppendorf tube and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. The supernatant was discarded and the pellet obtained at the bottom was washed in one millilitre of sterile triple distilled water by re-centrifugation at 10,000 rpm for 10 min at 4 ^{0}C. The pelleted cells obtained finally were resuspended in 300 microlitre of sterilized DNAs and RNAs free milliQ water. DNA template for PCR was prepared by boiling and snap chilling method. The mixture was boiled for 10 min. and then immediately chilled on crushed ice for 30 min. DNA samples extracted were stored at -20 °C for further use as template for PCR.

3.9.5. Setting up of PCR

PCR was performed in a total volume of 25 microlitre reaction mixture. A master mix was prepared before setting up of the PCR reaction by combining the following reagents in a 20 microlitre volume. The components of reaction mixture for one reaction were shown in table 4. Accordingly 540 microlitre master mix was prepared for 24 reactions.

To each PCR tube 22.5 µl of master mix and 2.5 µl of template DNA were added. One negative control without DNA and positive culture with template of MTCC 1143 were also added. The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme.

The cycling conditions included an initial denaturation followed by 35 cycles of final denaturation, 30 sec. annealing and 90 sec. extension. It was followed by final extension for 10 min. and held at 4 ^{0}C. The cyclic conditions used for the PCR are represented in the table 5. The whole reaction was conducted under the heated lid. The product was analysed by submarine agarose gel electrophoresis.

Table 4 . Components of reaction mixture

Sl. No.	**Name of the reagent**	**Quantity**
1	10X PCR buffer E	2.500 µl
2	15 mM $MgCl_2$	2.000 µl
3	Taq DNA polymerase	0.165 µl
4	dNTP	2.500 µl
5	Primer F	0.250 µl
6	Primer R	0.250 µl
7	Water	14.835 µl
Total		22.500 µl

3.9.6. Submarine Agarose Gel Electrophoresis

3.9.6.1. Method

The PCR product was detected by electrophoresis in 1.5 per cent agarose gel in TBE electrophoresis buffer (1X). TBE electrophoresis buffer 1X was prepared by diluting TBE 10X buffer with distilled water. Agarose was dissolved in TBE buffer (1X) by heating. When the mixture was cooled to around 50 ^{0}C, three microlitre of ethidium bromide was added. Melted agarose was then poured into clean, dry, gel platform, the edges of which were sealed with adhesive tape and the comb was kept in proper position. Once the gel was set, the comb and adhesive tape were removed gently and it was transferred to electrophoresis tank with wells kept towards cathode.

Table 5. Cyclic conditions used for PCR

Step	**Temperature**	**Time**
Initial denaturation	95 ^{0}C	2 min.
Final denaturation	95 ^{0}C	15 sec.
Annealing	60 ^{0}C	30 sec.
Extension	72 ^{0}C	90 sec.
Final extension	72 ^{0}C	10 min.

Amplified PCR product (five microlitre) was mixed with one microlitre of gel loading buffer and the samples were loaded in the wells. One well was set with 1.5 µl of 100bp ladder mixed with two microlitre of gel loading buffer. A reaction mix without DNA and one with *L. monocytogenes* MTCC 1143 total DNA were included as positive and negative controls respectively. Electrophoresis was carried out at 70V for one hour (or) until the bromophenol blue dye migrated to more than two third of the length of the gel.

The gel was visualized under UV transilluminator (Hoefer, USA) and the images were documented on gel documentation system (Bio- Rad Laboratories, USA).

3.10. ANTIBIOTIC SENSITIVITY

All *Listeria* isolates were subjected to antibiotic sensitivity test against 13 different antimicrobial agents by agar diffusion method, as per the procedure described by Bauer *et al.* (1966). Listeria isolates were tested against ampicillin (10µg), amoxycillin (30µg), cefotaxime (30µg), cefuroxime (30µg), chloramphenicol (30µg), cloxacillin (10µg), cotrimoxazole (25µg), doxycycline (30µg), erythromycin (15µg), gentamicin (10µg), rifampicin (5µg), streptomycin (25µg) and vancomycin (30µg) antibiotic discs (HiMedia).

3.10.1. Preparation of Mac Farland Standard

The turbidity standard solution was prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36 N H_2SO_4 (one per cent w/v). This solution is equal to half the density of No.1 Mac Farland standard solution. This solution was taken into glass tube, sealed tightly and kept in the dark, at room temperature for further use. The tube was vigorously agitated just before each use.

3.10.2. Preparation and Standardization of Inoculum

Three to four isolated colonies were selected from a pure culture and transferred into sterile nutrient broth and incubated at 37^0C, overnight. The turbidity of culture was adjusted using solution having half the density of Mac Farland standard No.1. When the broth culture was found to be more turbid, it was diluted with nutrient broth and when the turbidity was found to be less, culture was incubated for more time to achieve the required turbidity.

3.10.3. Inoculation

The swab was dipped into standardized inoculum and excess inoculum was removed from the swab by rotating it several times with a firm pressure on the inside wall of the test tube, above the fluid level. The Mueller Hinton agar

(HiMedia) plates were inoculated by swabbing over its entire surface, within 15 min. after adjusting the density of inoculum.

The swabbing procedure was repeated two more times, rotating the plates approximately 60^0 at each time, so as to ensure an even distribution of inoculums. The inoculum was allowed to dry for 15 min.

3.10.4. Application of Antibiotic Discs

The inoculated plate was left for not more than 15 min. at room temperature to absorb any excess surface moisture before applying the drug impregnated discs. The discs were applied to the surface of the inoculated agar with a sterile forceps. With the tip of the forceps, each disc was gently pressed down to ensure complete contact with the agar surface. During the application of discs care was taken not to place it closer than 15mm from the edge of the plate and the distance between the centre's of two such discs was not less than 24mm. The inoculated plate was inverted and incubated at 37 ^{0}C for 18 h., within 15 min. after the application of the discs.

3.10.5. Reading and Interpretation

At the end of the incubation period, the plates were examined and the diameter of the zones of complete inhibition was measured to the nearest whole millimeter with a scale held on the back of the Petri plate, which was illuminated with a reflected light.

The zone of inhibition of each disc was measured in three different directions keeping the midpoint of the disc as the centre of the zone. The mean of the measurement of inhibition was used for the interpretation of the results. The interpretation of the result was made by comparing diameter of the zone of inhibition with standard zone of inhibition chart provided by the disc manufacturing company. The clinical breakpoints for *Listeria* susceptibility testing were defined according to the Clinical and Laboratory Standard Institute

(CLSI, 2010) and the isolates were grouped as sensitive, intermediary sensitive and resistant, against each antibiotic.

3.11. STATISTICAL ANALYSIS

The data obtained were subjected to statistical analysis following procedure described by Snedecor and Cochran (1994) using the SPSS software version 17.

The significance of occurrence of *Listeria* spp. in beef, chicken and seafoods were assessed using single tailed ‘Z’ test.

Evaluation of immunoassay test and PCR was done by calculation of sensitivity, specificity, efficiency and predictive values (Martin, 1977). Definitions and calculations of these values are shown in table. 6. As the true *Listeria* spp. status of naturally contaminated samples is not known, the method of calculation assumes that the culture procedure is the true value. The efficiency of two tests was compared by calculation of the k coefficient.

The antibiotic sensitivity test results were subjected to hierarchial cluster analysis with dendrogram using average linkage (between groups) by rescaled distance cluster combine method.

Table 6. Definition and calculation of sensitivity, specificity, predictive value and k coefficient

		Culture procedure	
		+	-
Rapid test		**a**	**b**
		c	**d**

a- True positive, positive test with confirmed Listeria

b- False positive, positive test without confirmed Listeria

c- False negative, negative test with confirmed Listeria

d- True negative, negative test without confirmed Listeria

- Sensitivity (the ability to detect positive samples)- a/(a+c)
- Specificity (the ability to detect negative sample)- d/(b+d)
- Predictive value of:
  - Positive test (the probability of a positive result being correct)- a/(a+b)
  - Negative test (the probability of a negative result being correct)- d/(c+d)
- Kappa coefficient- (Efficiency-X)/(1-X)
  - Efficiency- (a+d)/(a+b+c+d)
  - X- [(a+b)/n][(a+c)/n)]+[(c+d)/n][(b+d)/n)]

Results

4. RESULTS

In the present investigation, occurrence of *Listeria* spp. in beef, chicken and seafoods were studied for a period of nine months from July, 2011 to March, 2012. The samples were collected aseptically from retail outlets in Mannuthy and Thrissur, local vendors and slaughter house at Thrissur. Isolation of *Listeria* spp. was carried out by conventional cultural technique. Further confirmation of the pathogenic species *L.monocytogenes* was carried out by immunological method using lateral flow technique and molecular method using Polymerase Chain Reaction. Antibiotic sensitivity test of the isolates were carried out to determine antibiotic of choice in treatment using standard disc diffusion method.

4.1. ISOLATION AND IDENTIFICATION OF *LISTERIA* SPECIES

A total of 385 samples consisting of 105 samples of fresh beef, 150 samples of raw chicken, 100 samples of prawn and 30 samples of sardines were collected from different sources. All the samples were subjected to isolation and identification of *Listeria* spp. by conventional culture technique. Species level differentiation was carried out by using carbohydrate utilization test and in vitro pathogenicity assay.

4.1.1 Beef

Fresh beef samples (105) were collected from retail outlets in Mannuthy and Thrissur, local vendors and slaughter house at Kuriachira, Thrissur and subjected to isolation and identification tests for determining Listeria organisms. The isolates were identified by cultural, morphological, biochemical and pathogenicity assays.

Out of the 105 samples screened, five samples had colonies with characteristics of *Listeria* spp. The *Listeria* spp. showed a characteristic gray green colour with a black sunken centre of about 0.5 mm diameter surrounded by a diffused black zone of aesculin hydrolysis on Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar (Figure 4 and 5). Identification of *Listeria* species on PALCAM agar plates was based on aesculin hydrolysis and mannitol fermentation. All *Listeria* species hydrolyse aesculin as evidenced by a blackening of the medium. Mannitol fermentation was demonstrated by a colour change in the colony and/or surrounding medium from red or gray to yellow due to the production of acidic end products.

The isolates showing characteristic colonies on PALCAM agar were then purified by subculturing and subjected to characterization of the organism by primary and secondary biochemical tests. All isolates obtained were gram positive, short rods or coccobacilli and motile at 20 to 25 ^{0}C. The biochemical characteristics of the isolates are given in the table 7.

All the five isolates from beef showed a uniform pattern of reaction to the biochemical tests thereby confirmed as *Listeria* spp. The isolates were then subjected to carbohydrate utilization tests (Table 1) for the identification of the organism at the species level (Fig. 6). The pattern of carbohydrate utilization by the Listeria isolates obtained from beef is shown in table 8.

Among the four isolates from beef except one (B2) all are dextrose and rhamnose positive suggesting that they are *L.monocytogenes*.

The isolate B2 is dextrose and mannitol positive but give negative reaction to the utilization of other sugars rhamnose and xylose and is suggestive of *L. grayi*.

The isolates were then subjected to pathogenicity test (Table 2) for further confirmation. The results of the pathogenicity assay of the isolates obtained from beef are shown in table 9.

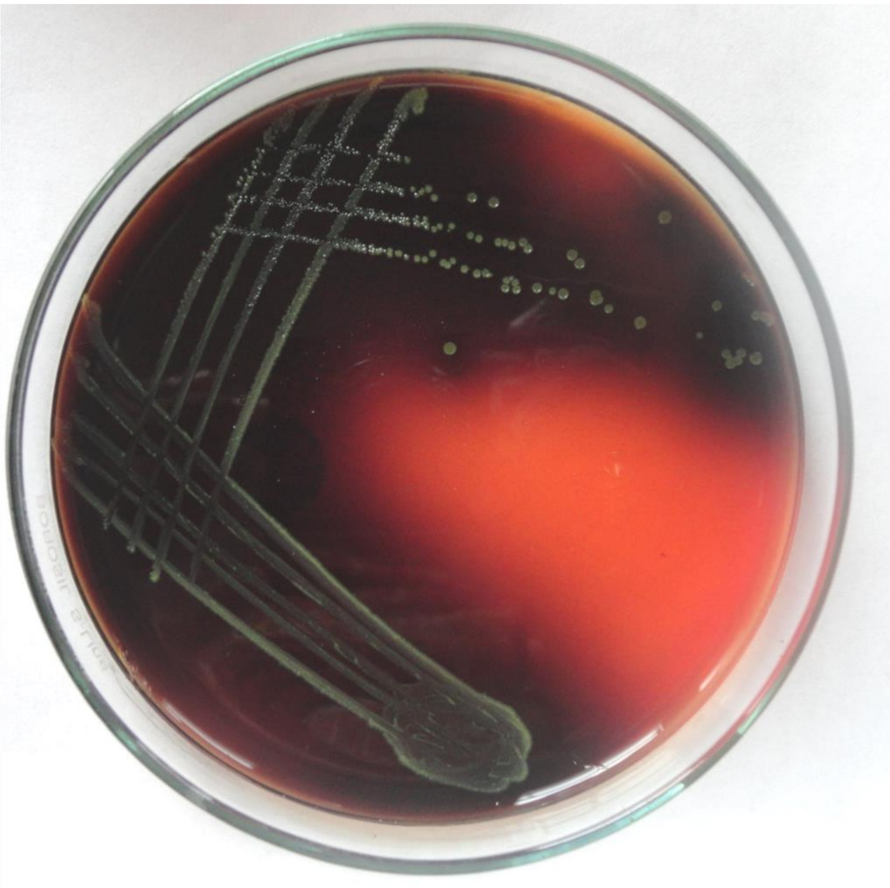

Fig. 4 ***Listeria*** **species on PALCAM agar**

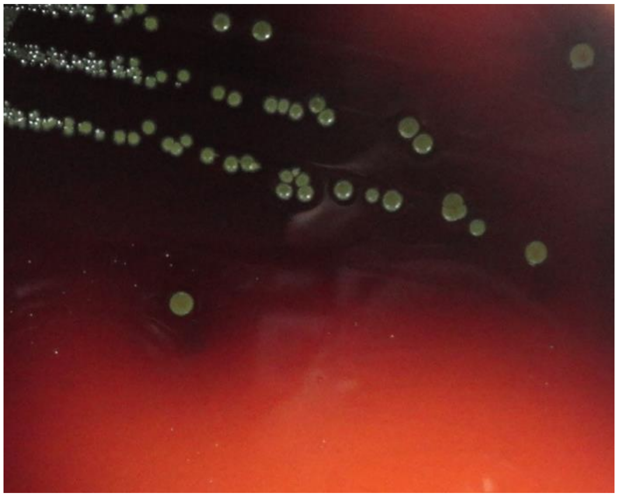

Fig. 5 Typical *L. monocytogenes* colony on PALCAM agar

Positive **Negative**

Fig. 6 Carbohydrate utilization pattern

Table 7. Biochemical characteristics of Listeria isolates obtained from beef

Biochemical Tests	**Listeria isolates**				
	B1	**B2**	**B3**	**B4**	**B5**
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
OF	F	F	F	F	F
MR	+	+	+	+	+
VP	+	+	+	+	+
Citrate utilization	-	-	-	-	-
Urease	-	-	-	-	-
Phosphatase	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+
Indole production	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-
Hippurate hydrolysis	+	+	+	+	+

F- Fermentative

Table 8. Carbohydrate utilization pattern of Listeria isolates obtained from beef

Sl. No.	**Acid from carbohydrates**	**Listeria isolates**				
		B1	**B2**	**B3**	**B4**	**B5**
1	D-mannitol	-	+	-	-	-
2	L-rhamnose	+	-	+	+	+
3	D-xylose	-	-	-	-	-
4	Dextrose	+	+	+	+	+

Table 9. Pathogenicity assays of Listeria isolates from beef

Pathogenicity test	Listeria isolates				
	B1	**B2**	**B3**	**B4**	**B5**
β hemolysis	+	-	+	+	+
CAMP test with *S.aureus*	+	-	+	+	+
CAMP test with *R. equi*	-	-	-	-	-

The pathogenicity assay revealed that out of the four isolates (B1, B3, B4 and B5) showed positive reaction to beta hemolysis and CAMP test with *S.aureus* and confirmed as *L.monocytogenes*. The isolate B2 was non beta hemolytic and showed negative reaction on CAMP test with both *S.aureus* and *R.equi* and hence identified as *L.grayi*.

4.1.2. Chicken

A total of 150 raw poultry samples were collected from retail outlets in Mannuthy and Thrissur and screened for *Listeria* spp. by conventional culture technique. All isolates showing specific colony characteristics of *Listeria* spp. on PALCAM agar were selected and subjected to characterization of the organism by primary and secondary identification tests. Out of 150 samples, four samples showed presence of *Listeria* spp.

All the four isolates were gram positive, short rods or coccobacilli and motile at 20 to 25 ^{0}C. The biochemical characteristics of the isolates obtained are given in the table 10.

The biochemical characterization of the isolates revealed a uniform pattern of reaction. The isolates showed hydrolysis of aesculin and hippurate and were of fermentative in nature thereby confirmed the generic level of the organism as Listeria.

Table 10: Biochemical test results of Listeria isolates from chicken

Biochemical tests	**Listeria isolates**			
	C1	**C2**	**C3**	**C4**
Catalase	+	+	+	+
Oxidase	-	-	-	-
OF	F	F	F	F
MR	+	+	+	+
VP	+	+	+	+
Citrate utilization	-	-	-	-
Urease	-	-	-	-
Phosphatase	+	+	+	+
Aesculin hydrolysis	+	+	+	+
Indole production	-	-	-	-
Gelatin hydrolysis	-	-	-	-
Hippurate hydrolysis	+	+	+	+

The biochemical characterization of the four isolates obtained from chicken namely C1, C2, C3 and C4 showed a uniform pattern suggestive of *Listeria* spp. The species level identification was carried out by carbohydrate utilization test. The carbohydrate utilization pattern of the isolates obtained from chicken is shown in table 11.

Table 11: Carbohydrate utilization pattern of Listeria isolates obtained from chicken

Sl. No.	Acid from carbohydrates	Listeria isolates			
		C1	C2	C3	C4
1	D-mannitol	-	-	-	-
2	L-rhamnose	+	+	+	-
3	D-xylose	-	-	-	+
4	Dextrose	+	+	+	+

Out of the four isolates of *Listeria* spp. obtained from chicken C1, C2 and C3 were able to produce acid only from dextrose and rhamnose suggesting that they were *L.monocytogenes*. The isolate C4 produced acid from dextrose and xylose only and was suggestive of *L.ivanovii*.

The four isolates were then subjected to in vitro pathogenicity assay for further confirmation of the species. The pathogenicity test results of isolates obtained from chicken is shown in the table 12.

Table 12. Pathogenicity assay of Listeria isolates from chicken

Pathogenicity assay	Listeria isolates			
	C1	C2	C3	C4
β hemolysis	+	+	+	++
CAMP test with *S.aureus*	+	+	+	-
CAMP test with *R. equi*.	-	-	-	+

The in vitro pathogenicity assay revealed that three out of four isolates (C1, C2 and C3) obtained from raw chicken were *L.monocytogenes* because they were beta hemolytic and showed positive CAMP test with *S.aureus*. The isolate

C4 produced a wider zone of beta hemolysis and showed positive reaction to CAMP test with *R. equi* and confirmed as *L.ivanovii*.

4.3. Seafoods

4.3.1. Prawn

A total of 100 samples of prawns of marine origin were collected from retail outlets and local vendors in Thrissur. Out of the 100 samples screened, 13 samples showed characteristic colonies on PALCAM agar. The typical colonies were selected and subjected to primary and secondary identification tests. Thirteen isolates were obtained from prawn. All the isolates were gram positive, short rods or coccobacilli and motile at 20 to 25^0C. The biochemical characteristics of the isolates obtained are given in the table 13.

All the thirteen isolates from prawns showed a uniform pattern of reaction to the biochemical tests confirming them as *Listeria* spp.

The isolates were then subjected to the carbohydrate utilization tests for the identification of the organisms at species level. The results of the carbohydrate utilization test are given in Table 14.

Among the 13 isolates of Listeria organism subjected to carbohydrate utilization test two (S5 and S6) were mannitol and dextrose positive, suggestive of *L.grayi*. All the other isolates produced acid by utilizing rhamnose and dextrose suggesting that they belong to the pathogenic species *L.monocytogenes.*

Table 13. Biochemical test results of Listeria isolates from prawn

Biochemical tests	Listeria isolates												
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**	**S11**	**S12**	**S13**
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
OF	F	F	F	F	F	F	F	F	F	F	F	F	F
MR	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-
Phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-
Hippurate hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 14. Carbohydrate utilization pattern of Listeria isolates obtained from prawn

Sl. No	Acid from carbohydrates	Listeria isolates												
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
1	D-mannitol	-	-	-	-	+	+	-	-	-	-	-	-	-
2	L-rhamnose	+	+	+	+	-	-	+	+	+	+	+	+	+
3	D-xylose	-	-	-	-	-	-	-	-	-	-	-	-	-
4	Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 15. Pathogenicity assay of Listeria isolates from prawn

Pathogenicity assay	Listeria isolates												
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
β hemolysis	+	+	+	+	-	-	+	+	+	+	+	+	+
CAMP test with *S.aureus*	**+**	**+**	**+**	**+**	**-**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
CAMP test with *R. equi*	-	-	-	-	-	-	-	-	-	-	-	-	-

Further confirmation of the isolates was carried out by pathogenicity assay. The non beta hemolytic colonies and negative reaction to the CAMP test by both S5 and S6 indicated that they belong to the same species *L.grayi*. All the remaining 11 isolates produced zone of hemolysis on the blood agar plate and enlarged zone of hemolysis with *S.aureus* on CAMP test and were confirmed as *L.monocytogenes* (Table 15).

4.3.2. Sardines

Thirty samples of sardines were collected from retail outlets in Thrissur and subjected to isolation and identification of *Listeria* spp. All the samples screened were free of *Listeria* spp.

4.4. Overall prevalence of *Listeria* spp. in Foods of Animal Origin

The isolation and identification of *Listeria* spp. were carried out from three categories of foods of animal origin: beef, chicken and seafood. The samples were collected from retail outlets, supermarkets, local vendors and the slaughter house at Kuriachira in Thrissur. The occurrence of *Listeria* spp. in beef, chicken and seafoods by conventional culture technique is shown in table 16.

Table 16. Occurrence of *Listeria* species in foods of animal origin

Food samples	Samples screened	*Listeria* spp.		*L.monocytogenes*		*L.ivanovii*		*L.grayi*	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Beef	105	5	4.76	4	3.81	-	-	1	0.95
Chicken	150	4	2.67	3	2.0	1	0.67	-	-
Prawn	100	13	13.0	11	11.0	-	-	2	2.0
Sardine	30	-	-	-	-	-	-	-	-
Total	**385**	**22**	**5.71**	**18**	**4.67**	**1**	**0.26**	**3**	**0.78**

Out of 385 samples analysed by the conventional cultural techniques, 22 (5.71 per cent) samples were positive for *Listeria* spp. and *L.monocytogenes* were detected in 18 samples (4.67 per cent). *L.monocytogenes* were present in four (3.81 per cent), three (two per cent) and 11 (11.0 per cent) samples of beef, chicken and prawns respectively. Occurrence of *L. monocytogenes* in foods of animal origin is represented in the figure 7. Four isolates of other *Listeria* spp. were obtained from beef, chicken and seafoods. *L.grayi* was detected in one beef and two prawn samples. One sample of chicken was positive for *L.ivanovii* (0.26 per cent). Thirty samples of sardines screened were negative for the presence of *Listeria* spp. The prevalence of *Listeria* spp. in foods of animal origin screened is depicted in the figure 8.

The statistical significance of the occurrence of *Listeria* spp. in beef, chicken and seafoods were calculated by test for proportion using single tailed 'Z' test. The Z value indicated that the occurrence in beef, chicken and seafoods were statistically significant.

4.2. IMMUNOLOGICAL TECHNIQUE

Lateral flow assay based on the immune flow principle was used for the qualitative detection and confirmation of *L.monocytogenes* in food. Singlepath L'mono, an immunochromatographic rapid test based on gold labeled antibodies was used for the purpose. (Figure 9)

All the isolates of *Listeria* spp. obtained by conventional culture technique were assayed for confirming the presence of *L.monocytogenes*. Twenty two isolates of *Listeria* spp. from beef, chicken and seafoods were subjected to lateral flow assay. Out of these 22 isolates, 13 were positive for *L.monocytogenes* by lateral flow technique.

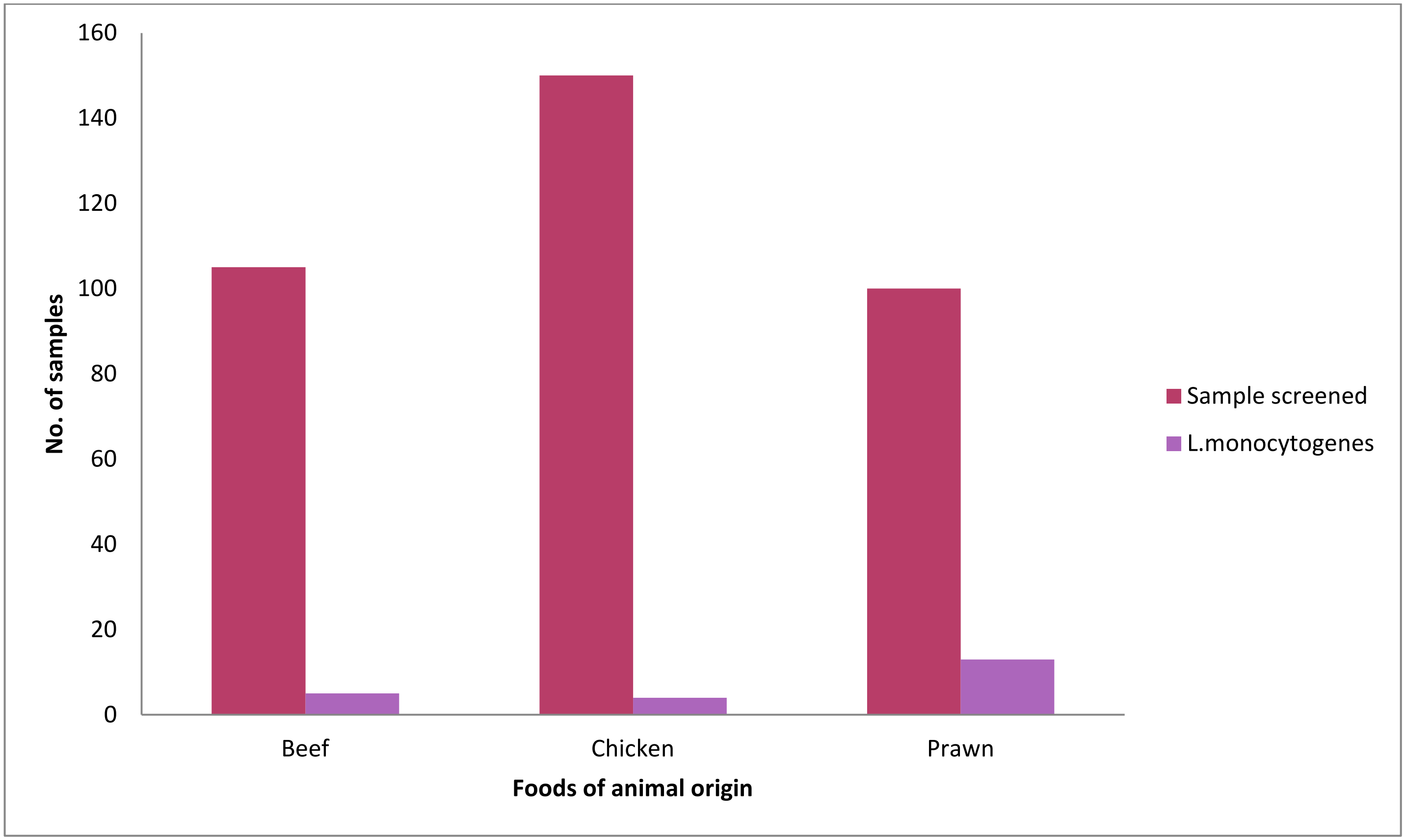

Fig. 7. Occurrence of *L.monocytogenes* in foods of animal origin

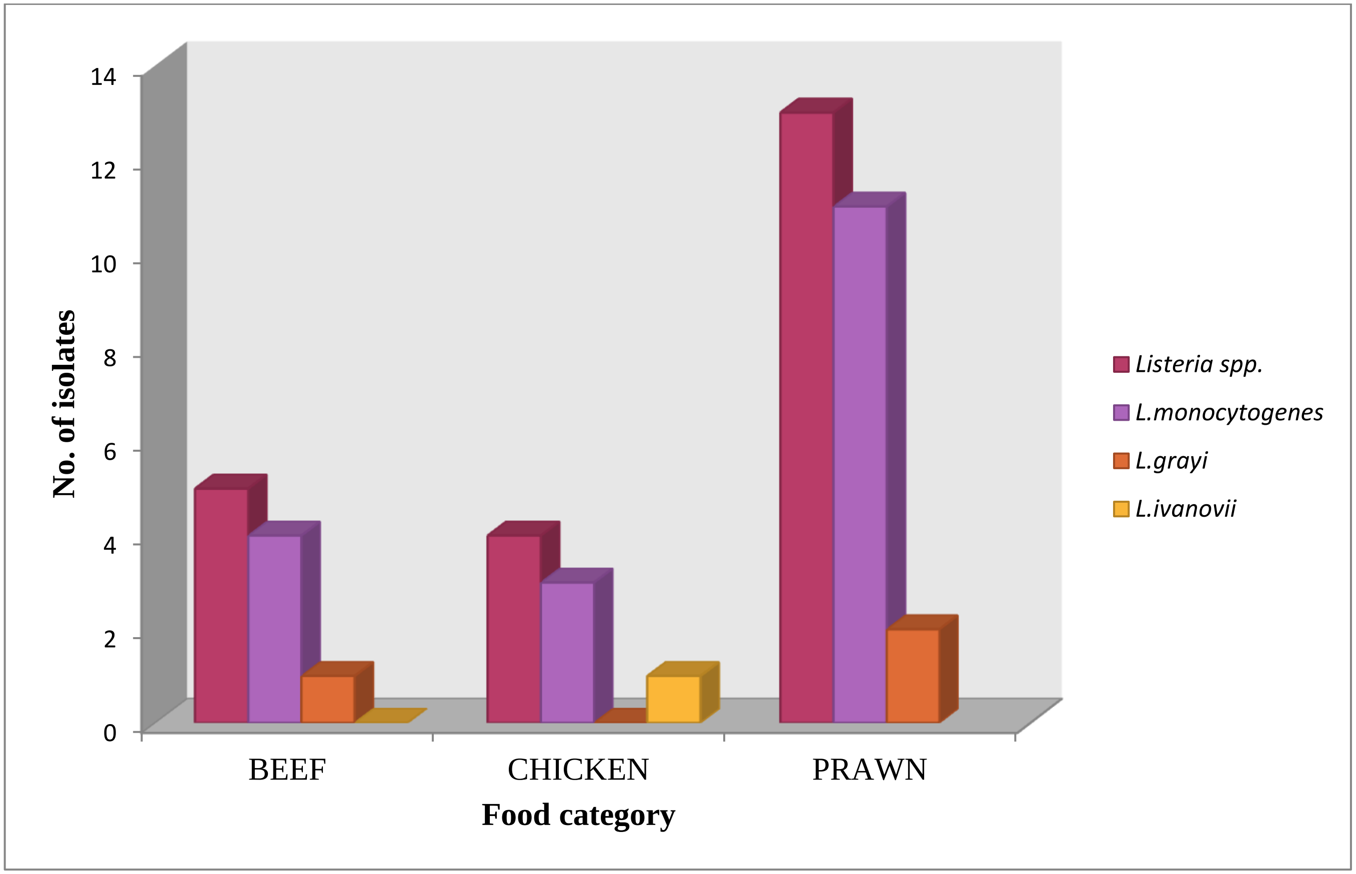

Figure 8. Occurrence of *Listeria* species in foods of animal origin

Positive **Negative**

Fig. 9 Lateral Flow Assay

4.2.1. Comparison of Lateral Flow Assay with Culture Technique

The results of detection of *L.monocytogenes* by lateral flow assay and conventional culture technique are depicted in table 17.

Table 17. *L.monocytogenes* detection by Lateral flow assay and conventional culture technique from foods of animal origin

Food samples	**No. of Isolates screened**	**Samples positive for *L.monocytogenes***	
		By culture technique	**By Lateral flow assay**
Beef	5	4	2
Chicken	4	3	2
Prawn	13	11	9
Total	22	18	13

Among five isolates of *Listeria* spp. obtained from beef, four (B1, B3, B4 and B5) were positive for *L.monocytogenes* by culture technique and B1 and B3 confirmed as *L.monocytogenes* by lateral flow assay.

In case of chicken among four isolates screened, C1, C2 and C3 were positive for *L.monocytogenes* by conventional culture technique where as C1 and C2 were found positive by lateral flow assay.

Thirteen isolates (S1 to S13) of prawn were subjected to conventional culture technique and lateral flow assay for confirming *L.monocytogenes*. Except S5 and S6 all others were confirmed as *L.monocytogenes* by culture technique. Nine isolates (S3, S4, S7, S8, S9, S10, S11, S12 and S13) were positive for the organism by lateral flow technique.

Two, two and nine samples of beef, chicken and prawns respectively were positive for *L.monocytogenes* by lateral flow technique. Overall 13 isolates were positive for *L.monocytogenes* by immunological method.

Evaluation of lateral flow assay was carried out by calculation of sensitivity, specificity and positive and negative predictive values. As the true *Listeria* spp. status of naturally contaminated samples is not known, the method of calculation assumed that the culture procedure as the true value. The results are given in table 18.

Table 18. Statistical evaluation of Lateral flow assay with conventional culture technique

Listeria singlepath L'mono test result	Culture method		Sensitivity (%)	Specificity (%)	Predictive value (%)		Kappa coefficient
	+	-			+	-	
+	13(a)	0 (b)	72.22	100	100	44.44	0.49
-	5 (c)	4 (d)					

The comparative study showed that the lateral flow assay has a specificity of 100 per cent. Specificity denotes the ability to detect negative samples. The sensitivity is the ability to detect positive samples and the kit has 72.22 per cent sensitivity. Predictive value of 100 per cent in positive test is the probability of a positive result being correct and 44.44 per cent of negative test explained the probability of a negative result being correct.

4.3. POLYMERASE CHAIN REACTION

All the Listeria isolates obtained from foods of animal origin were subjected to polymerase chain reaction. The oligonucleotide primer targeting the listeriolysin O gene (*hly*A) was used for detecting *L.monocytogenes*. The standardized PCR allowed the amplification of virulence associated gene of *L.monocytogenes* namely *hly*A to their respective base pair, 456 bp. The PCR product was represented by a single band in the corresponding region of the DNA marker ladder (Fig. 10).

The twenty two isolates of *Listeria* spp. obtained by conventional culture technique from beef, chicken and seafood were subjected to PCR for confirming the presence of *L.monocytogenes*. Out of 22 isolates, sixteen were positive for *L.monocytogenes* by PCR technique.

4.3.1. Comparison of Conventional Culture Technique with PCR

The isolates confirmed by carbohydrate utilization tests and in vitro pathogenicity tests were subjected to PCR for identification of *L.monocytogenes* by detecting the virulence gene *hly* A. The isolation rate of *L.monocytogenes* from beef, chicken and prawn by PCR and culture method are represented in the table19.

Table 19. *L.monocytogenes* detection by PCR and conventional culture technique from foods of animal origin

Food sample	**No. of isolates screened**	**No. of samples positive for *L.monocytogenes***	
		By culture method	**By PCR**
Beef	5	4	4
Chicken	4	3	2
Prawn	13	11	10
Total	22	18	16

Five isolates of *Listeria* spp. obtained from beef were subjected to confirmation by biochemical tests, pathogenicity assay and by PCR. The isolates B1, B3, B4 and B5 were confirmed as *L.monocytogenes* by conventional culture method as well as by molecular method.

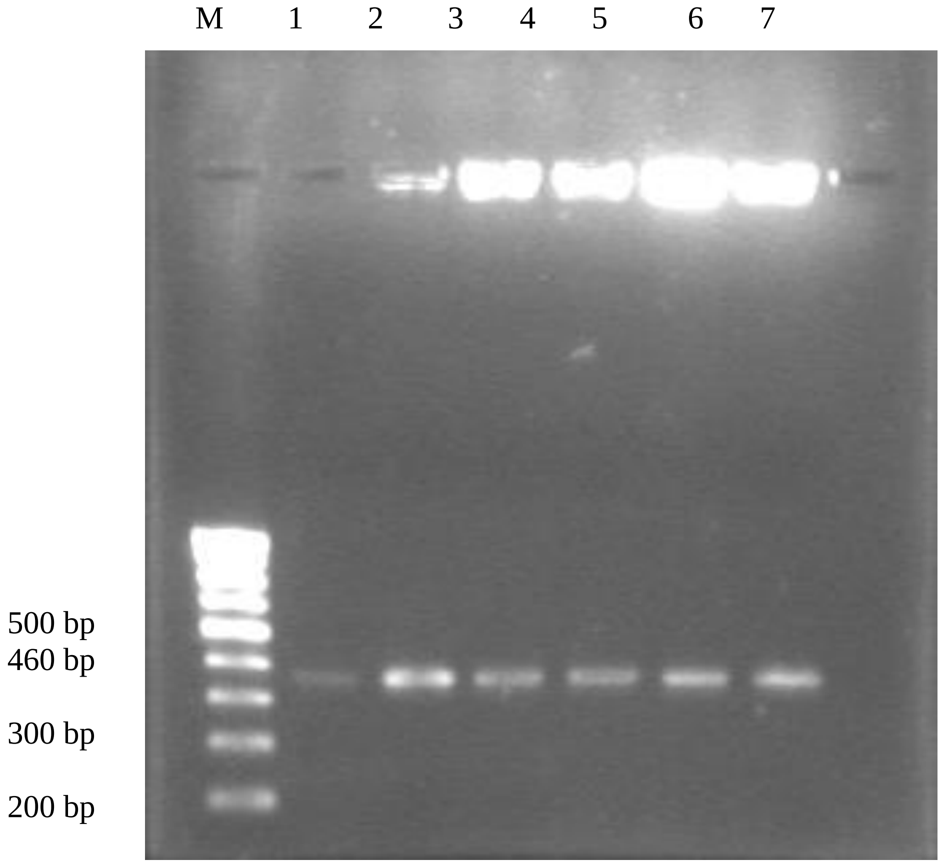

Fig. 10. PCR profile of *Listeria monocytogenes* isolates

Lane M: 100 bp ladder

Lane 1-5: *L.monocytogenes* isolates from foods of animal origin

Lane 6: *L.monocytogenes* MTCC 1143 (Positive control)

Lane 7: Negative control

In the case of prawn eleven (except S5 and S6) out of 13 were positive for *L.monocytogenes* by culture technique whereas ten excluding S1, S5 and S6 by PCR.

L.monocytogenes was detected in four samples of beef, two samples of chicken and ten prawn samples by PCR. Overall sixteen isolates were confirmed by PCR among 22 screened for the presence of *L.monocytogenes*.

4.3.2. Comparison of Lateral Flow Assay with PCR

The results of detection of *L.monocytogenes* by Lateral flow assay and PCR from beef, chicken and seafood are depicted in the table 20.

Table 20. *L.monocytogenes* detection by Lateral Flow Assay and PCR

Food sample	**No. of isolates screened**	**No. of samples positive for *L.monocytogenes***	
		By Lateral flow assay	**By PCR**
Beef	5	2	4
Chicken	4	2	2
Prawn	13	9	10
Total	22	13	16

Among five isolates of *Listeria* spp. obtained from beef, two (B1 and B3) were positive for *L.monocytogenes* by lateral flow assay whereas B1, B3, B4 and B5 were confirmed as *L.monocytogenes* by PCR.

In case of chicken C1 and C2 were confirmed as *L.monocytogenes* by lateral flow assay and PCR among four isolates screened.

Thirteen isolates of prawn obtained were subjected to lateral flow assay and PCR for confirming *L.monocytogenes*. Nine isolates (S3, S4, S7, S8, S9, S10, S11, S12 and S13) were positive for the organism by lateral flow technique where as ten isolates (S2, S3, S4, S7, S8, S9, S10, S11, S12 and S13) were showed positive reaction to PCR for the detection of the organism.

Two, two and nine samples of beef, chicken and prawns respectively were positive for *L.monocytogenes* by lateral flow technique and four, two and ten by PCR.

Evaluation of PCR was carried out in the similar way as that for lateral flow technique by calculation of sensitivity, specificity and positive and negative predictive values. As the true *Listeria* spp. status of naturally contaminated samples is not known, the method of calculation assumed that the culture procedure as the true value. The results are given in table 21.

Table 21. Statistical evaluation of PCR with conventional culture technique

PCR	Culture method		Sensitivity (%)	Specificity (%)	Predictive value (%)		Kappa coefficient
	+	-			+	-	
+	16(a)	0 (b)	88.89	100	100	66.67	0.75
-	2(c)	4(d)					

The comparative study showed that the PCR has a specificity of 100 per cent. Specificity denotes the ability to detect negative samples. The sensitivity is the ability of the method to detect positive samples and PCR has a sensitivity of 88.89 per cent. Predictive value of 100 per cent in positive test indicated the probability of a positive result being correct and 66.67 per cent of negative test explained the probability of a negative result being correct.

4.4. ANTIBIOTIC SENSITIVITY TEST

All the 22 isolates of *Listeria* spp. obtained from three categories of food samples viz; beef, chicken and seafoods were subjected to antibiotic sensitivity test by standard disc diffusion method. The sensitivity pattern was assessed using the disk diffusion assay, according to the criteria defined by the Clinical Laboratory Standard Institute (CLSI) recommended by the World Health Organization. (Figure 11)

4.4.1. The antibiotic sensitivity pattern based on food of animal origin

The antibiotic sensitivity pattern of the five isolates of *Listeria* spp. obtained from beef, four from chicken and thirteen from prawn were screened by Bauer Kirby disc diffusion method. It is useful in determining the antibiotic of choice when suspecting a foodborne infection from particular source. The antibiotic sensitivity pattern of the isolates from different food samples is summarized in the table 22.

All the isolates of *Listeria* spp. from beef, chicken and seafoods were sensitive to cotrimoxazole, doxycycline, erythromycin, gentamicin and vancomycin. The antibiotic sensitivity pattern of *Listeria* spp. is represented in the figure 12. The zone of inhibition was maximum for doxycycline. The isolates from chicken and seafoods were also sensitive to chloramphenicol and streptomycin. The isolates of *Listeria* spp. obtained from beef, chicken and seafoods were resistant to ampicillin and cloxacillin.

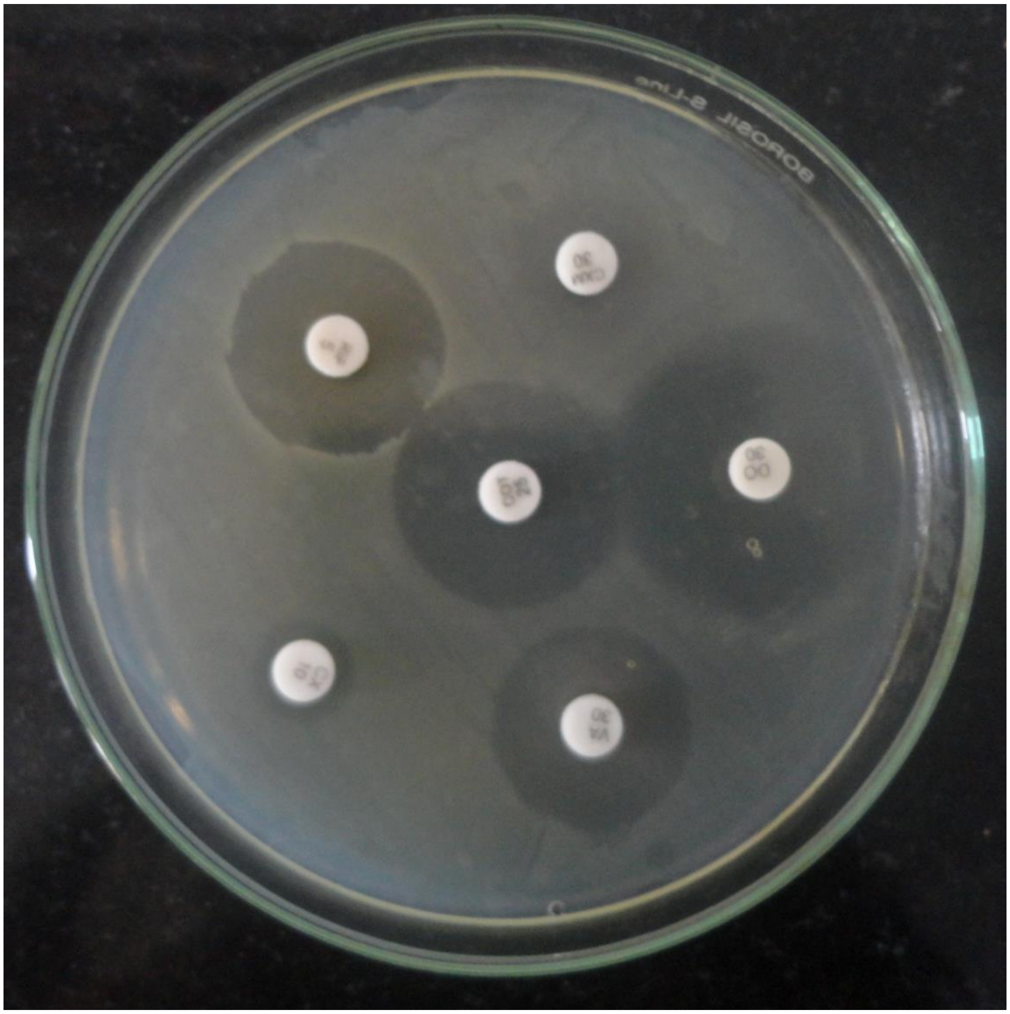

Fig. 11 Antibiogram of Listeria isolates

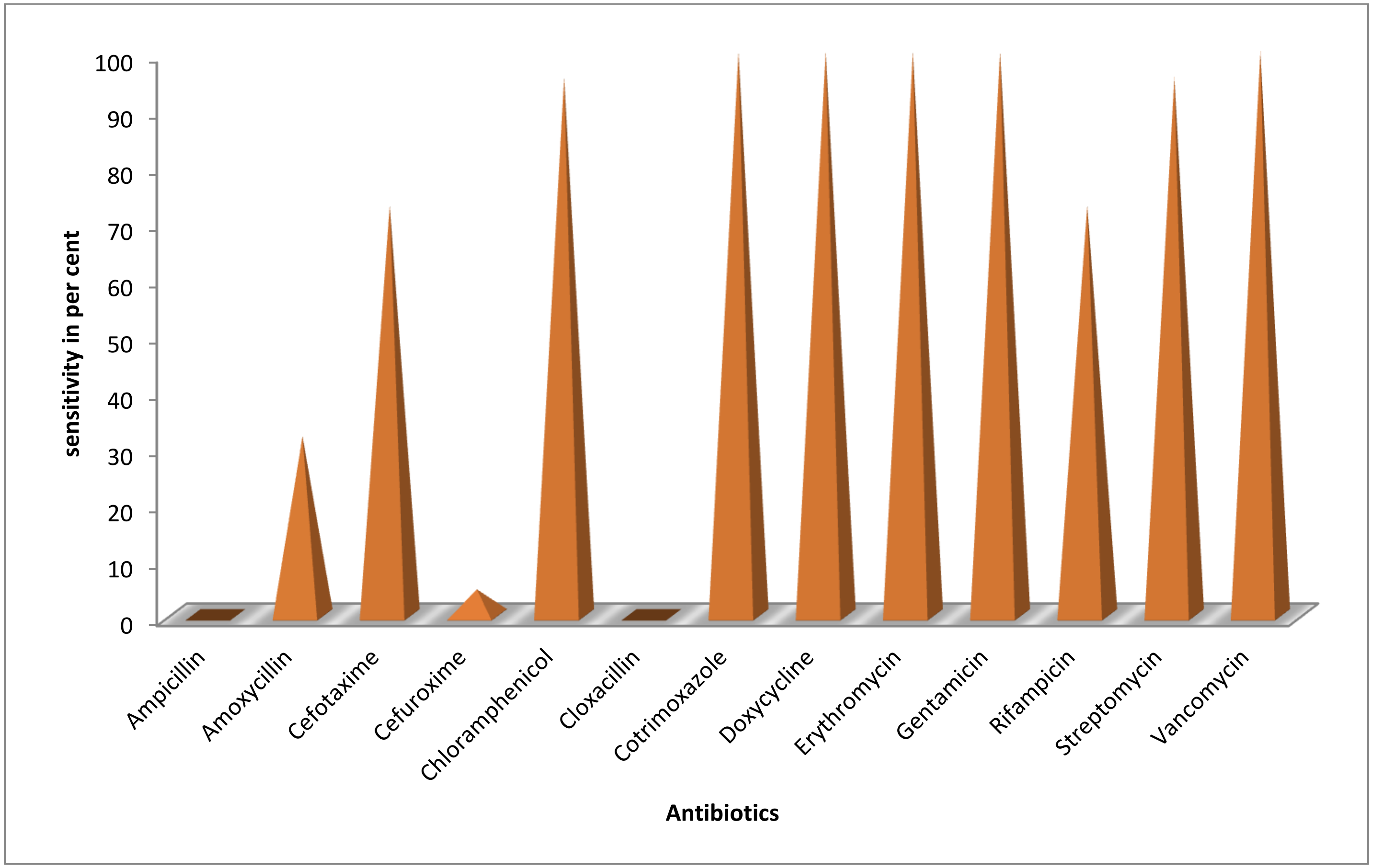

Fig. 12 Antibiotic sensitivity pattern of *Listeria* isolates from foods of animal origin

Table 22. Antibiotic sensitivity pattern based on food of animal origin

Antibiotic (μg/ disc)	Beef (5 isolates)			Chicken (4 isolates)			Seafood (18 isolates)		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Ampicillin (10)	5	-	-	4	-	-	13	-	-
Amoxycillin (30)	5	-	-	2	-	2	8	-	5
Cefotaxime (30)	-	3	2	1	1	2	-	1	12
Cefuroxime (30)	3	2	-	1	2	1	8	5	-
Chloramphenicol (30)	-	1	4	-	-	4	-	-	13
Cloxacillin (10)	5	-	-	4	-	-	13	-	-
Cotrimoxazole (25)	-	-	5	-	-	4	-	-	13
Doxycycline (30)	-	-	5	-	-	4	-	-	13
Erythromycin (15)	-	-	5	-	-	4	-	-	13
Gentamicin (10)	-	-	5	-	-	4	-	-	13
Rifampicin (5)	1	1	3	-	-	4	-	4	9
Streptomycin (25)	1	-	4	-	-	4	-	-	13
Vancomycin (30)	-	-	5	-	-	4	-	-	13

R- resistant I- Intermediate S- sensitive

Among the isolates of *Listeria* obtained from beef, three isolates (B1, B3 and B4) showed resistance to cefuroxime, and only B4 was resistant to rifampicin and streptomycin. The isolates from beef showed complete resistance to amoxicillin. None of the isolates from beef were resistant to cefotaxime and chloramphenicol where as only B1, B2 and B3 were sensitive to rifampicin and B1, B2, B4 and B5 were sensitive to chloramphenicol.

The isolates C2 and C3 from chicken were sensitive to amoxycillin and cefotaxime and only C1 to cefuroxime. All the four isolates of Listeria spp. from chicken were sensitive to rifampicin.

All the isolates from prawns except S9 were sensitive to cefotaxime. Five isolates namely S1, S2, S3, S6 and S8 were sensitive to amoxycillin and none of the isolates were sensitive to cefuroxime. Except four isolates viz; S2, S4, S12 and S13 all isolates were resistant to rifampicin.

All the isolates showed resistance to more than one antibiotic (multiple resistance). The maximum multiple resistance was shown by an isolate from beef, B4. It showed resistance to six antibiotics namely ampicillin, amoxycillin, cloxacillin, cefuroxime, rifampicin and streptomycin.

4.4.2. The antibiotic sensitivity pattern of individual *Listeria* species

The *Listeria* spp. isolated from beef, chicken and seafoods were 18 isolates of *L.monocytogenes*, three *L.grayi* and one *L.ivanovii*. The antibiotic sensitivity pattern of the different species of *Listeria* organisms to 13 commonly used antibiotics was studied and is represented in the table 23. The antibiotic sensitivity pattern of *L.monocytogenes* is depicted in figure 13.

All the isolates of *L.monocytogenes, L.ivanovii* and *L.grayi* showed sensitivity to cotrimoxazole, doxycycline, erythromycin, gentamicin and vancomycin. The isolates of *L.ivanovii* and *L.grayi* were also sensitive to chloramphenico, rifampicin and streptomycin. None of the isolates of *Listeria* spp. were sensitive to ampicillin and cloxacillin. *L.ivanovii* isolate was also resistant to amoxycillin.

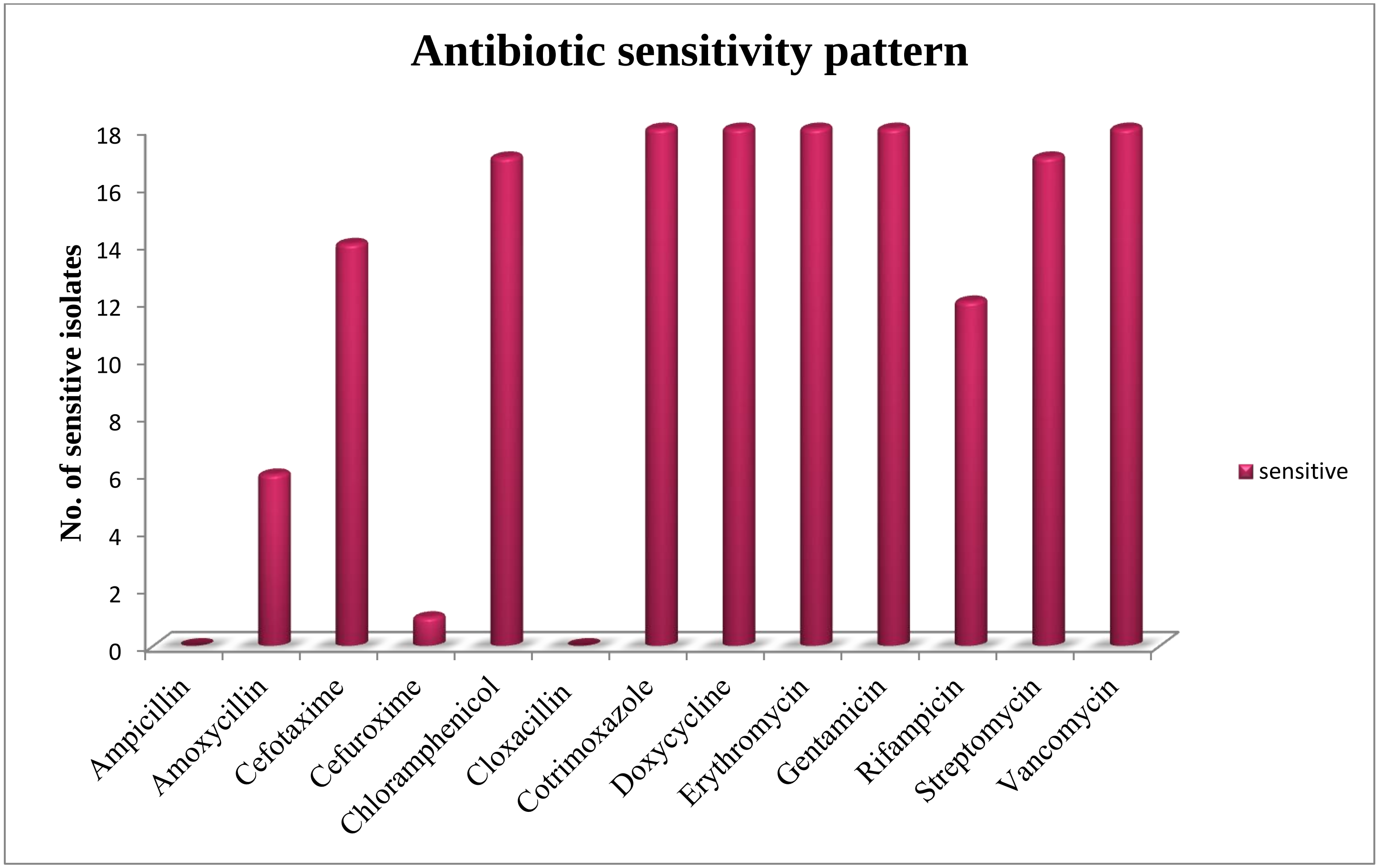

Fig. 13 Antibiotic sensitivity pattern of *L.monocytogenes*

Except B3 all other 17 *L.monocytogenes* isolates were sensitive to chloramphenicol and all other except B4 were sensitive to streptomycin. Only C1 showed sensitivity to cefuroxime where as resistance to cefotaxime. Among 22 isolates fourteen were sensitive to cefotaxime and six isolates were sensitive to amoxicillin. Only B4 showed resistance to rifampicin. Among three isolates of *L.grayi*, S5 and S6 were sensitive to cefotaxime whereas only S6 to amoxycillin.

Table 23. The antibiotic sensitivity pattern of different *Listeria* species

Antibiotic (µg/ disc)	***L.monocytogenes* (18 isolates)**			***L.ivanovii* (1 isolates)**			***L.grayi* (3 isolates)**		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Ampicillin (10)	18	-	-	1	-	-	3	-	-
Amoxycillin (30)	12	-	6	1	-	-	2	-	1
Cefotaxime (30)	1	3	14	-	1	-	-	1	2
Cefuroxime (30)	11	6	1	-	1	-	1	2	-
Chloramphenicol (30)	-	1	17	-	-	1	-	-	3
Cloxacillin (10)	18	-	-	1	-	-	3	-	-
Cotrimoxazole (25)	-	-	18	-	-	1	-	-	3
Doxycycline (30)	-	-	18	-	-	1	-	-	3
Erythromycin (15)	-	-	18	-	-	1	-	-	3
Gentamicin (10)	-	-	18	-	-	1	-	-	3
Rifampicin (5)	1	5	12	-	-	1	-	-	3
Streptomycin (25)	1	-	17	-	-	1	-	-	3
Vancomycin (30)	-	-	18	-	-	1	-	-	3

R- Resistant I- Intermediate S- Sensitive

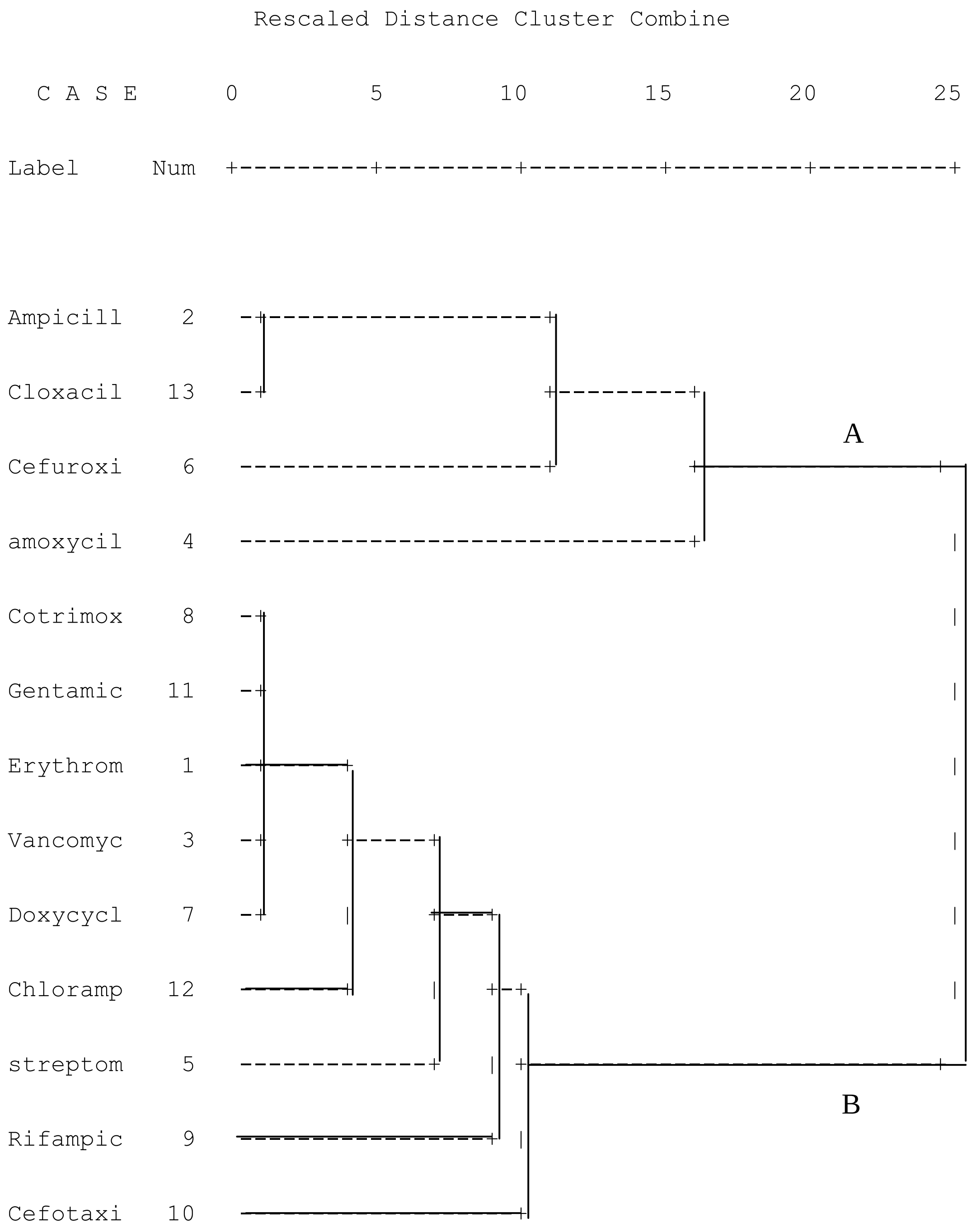

Fig. 14. Dendrogram using Average Linkage (Between Groups) for *Listeria* species

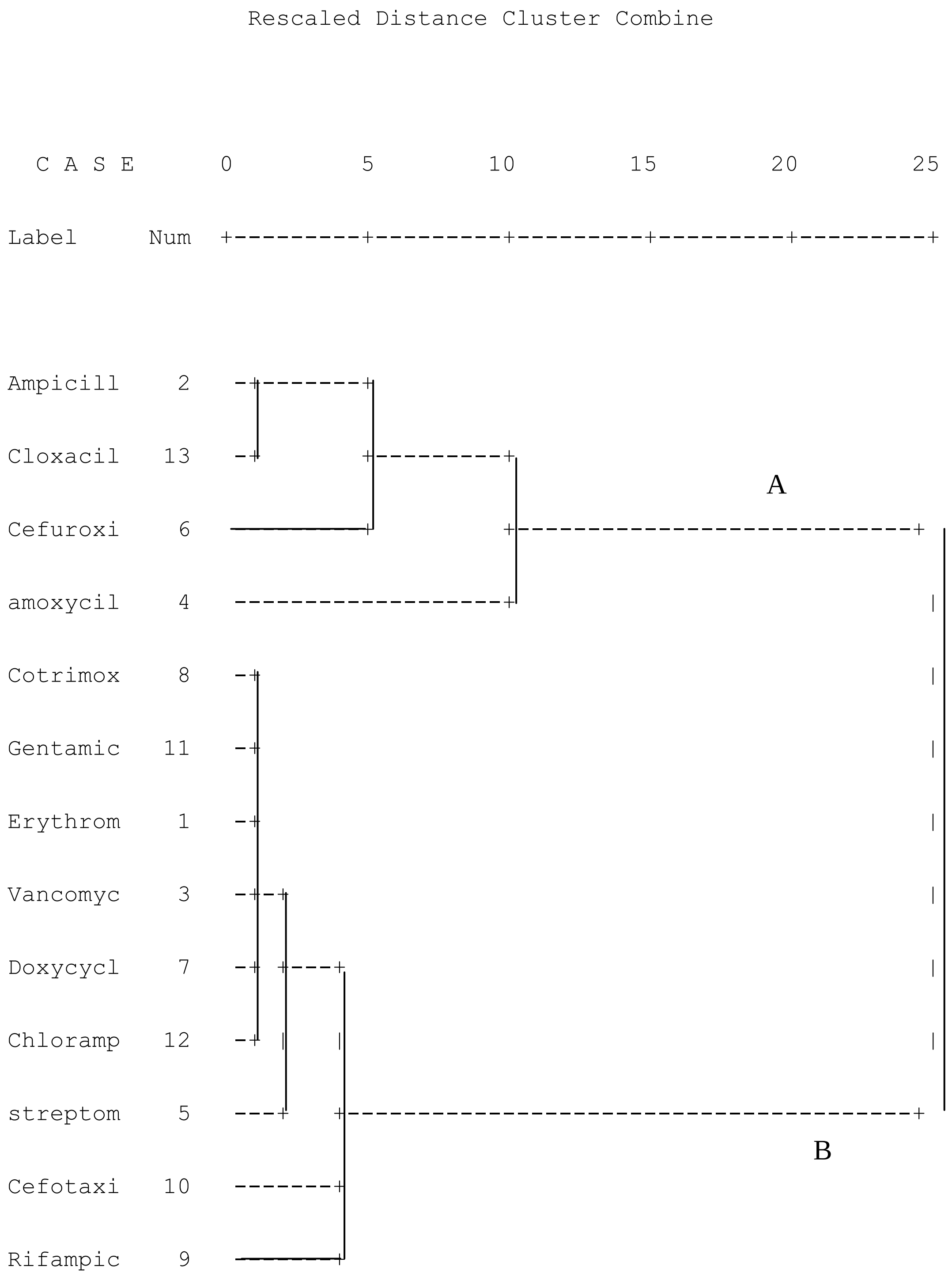

Figure 15. Dendrogram using Average Linkage (Between Groups) for *L.monocytogenes*

The dendrogram based on antimicrobial drug sensitivity pattern of Listeria isolates is represented in the figure 14. The procedure used for combining clusters is ‘between group linkage’ based on rescaled distance cluster combine. The accessions are clearly divided into two major clusters A and B. The maximum dissimilarity between the clusters A and B is 25 units. The groups that are closest with maximum similarity is that formed at single unit distance by ampicillin and cloxacillin and another big cluster formed by cotrimoxazole, gentamicin, erythromycin, vancomycin and doxycycline. The former is the cluster which are resistant to all isolates under study and the latter group sensitive to all.

The dendrogram of antibiotic sensitivity pattern of isolates of pathogenic species *L.monocytogenes* is depicted in the figure 15. The maximum dissimilarity between the clusters A and B is 25 units. In this the big cluster formed by the addition of one more antibiotic chloramphenicol in addition to those antibiotics sensitive to all Listeria isolates.

Discussion

5. DISCUSSION

The prevalence of foodborne illnesses in both industrialized as well as in non- industrialized countries is increasing. It may be due to major changes in food production, preservation, storage and consumption. Globalization of food trade and import of foods also play a role in occurrence of foodborne diseases. Listeriosis is an emerging foodborne disease caused by pathogenic *Listeria monocytogenes*.

L. monocytogenes is a common inhabitant of the gastro-intestinal tract of animals and man. Contaminated manure can introduce the pathogen to feed and the natural environment. *Listeria* species are ubiquitous in nature and can survive in a wide range of temperature, pH and tolerate osmotic stress and carbon starvation. Hence it can easily contaminate various raw and processed foods, including milk and dairy products, meat, fresh vegetables as well as seafood and fish products (Yucel *et al.*, 2005). Foods can be contaminated during the primary production phase or during processing or at distribution. Human infection occurs mainly due to consumption of contaminated food (Minea *et al.*, 2005).

Listeria has been involved in a large number of foodborne outbreaks all over the world. The most hazardous products are those ready to eat which are stored at room temperature for a long period of time. *Listeria* spp. has been isolated from poultry, red meat and meat products, seafoods in many countries around the world, although these foods have not been associated with documented outbreaks of listeriosis in India.

A total of 385 samples of fresh beef, raw chicken and seafoods collected from retail outlets in and around Thrissur were examined throughout for a nine month sampling period. The isolates of *Listeria* species obtained were then subjected to immunological method using lateral flow assay and molecular method using PCR for confirming *L.monocytogenes*. The screening of food samples for the isolation of *Listeria* species, technique of confirmation and antibiotic sensitivity pattern of the isolates are discussed under following titles.

5.1. ISOLATION AND IDENTIFICATION OF *LISTERIA* SPECIES

The occurrence of *Listeria* spp. in beef, chicken and seafoods collected from retail outlets in Mannuthy and Thrissur, local vendors and slaughter house at Kuriachira, Thrissur were determined by conventional culture technique. The identification of the isolates was carried out by primary and secondary biochemical tests. The species level confirmation was based on carbohydrate utilization pattern and in vitro pathogenicity assay.

There are several official, standardized protocols designed by regulatory agencies and organizations for *Listeria* spp. enrichment and isolation. The commonly using protocols are that of Food and Drug Administration (FDA), The United States Department of Agriculture- Food Safety Inspection Service (USDA- FSIS) and International organization for Standardisation (ISO).

The procedure followed in the study was USDA method described by Ryser and Donnelly (2001) with necessary modifications put forward by Parihar *et al*., 2008. The procedure consisted of two step enrichment followed by selective plating on PALCAM agar.

The primary enrichment was carried out in University of Vermont Medium I (UVM I). Enrichment works on a Darwinian concept to shift the balance in the environment to favour the organism of interest over the other bacteria present in the sample. The selective compounds in UVM I are acriflavin and nalidixic acid. Acriflavin is a toxic dye which inhibits the growth of faecal streptococci. It works well by inhibiting many gram positive cocci, including *Staphylococcus aureus* and some lactic acid bacteria. Nalidixic acid inhibits many species of gram negative bacteria. It blocks DNA replication in susceptible bacteria by acting on DNA gyrase.

The secondary enrichment broths contain higher concentration of antibiotics and dyes to allow more robust selection of *Listeria* spp. The secondary enrichment was done in UVM II which contains acriflavin and nalidixic acid at a higher concentration.

The plating was done on PALCAM agar after selective enrichment. The selectivity of the PALCAM medium is achieved through the presence of lithium chloride, polymyxin B sulphate and acriflavin hydrochloride present in the medium base and ceftazidime provided by PALCAM antimicrobial supplement. These agents effectively suppress growth of most commonly occurring non-*Listeria* species of bacteria present in food samples. The polymyxin B disrupts the cell membrane of gram negative organisms. It is particularly effective against *Pseudomonas* spp. Lithium chloride make use of the salt tolerance capacity of *Listeria* spp., and also serves to inhibit enterococci and some gram negative bacteria.

The identification of the organism was carried out based on biochemical tests (Barrow and Feltham, 1993). Species level identification was done based on carbohydrate utilization test and in vitro pathogenicity assay.

The occurrence of *Listeria* spp. in beef, chicken and seafoods are discussed below.

5.1.1. Beef

Of the 105 fresh beef samples screened, the prevalence rate of *Listeria* spp. was 4.76 per cent. *L.monocytogenes* and *L.grayi* was detected in 3.81 per cent and 0.95 per cent of beef samples respectively. The findings of the present study are in accordance with the study of Mengesha *et al.*, 2009 in Ethiopia and Wagner *et al.,* 2007 in Austria.

The prevalence of *Listeria* spp. may be due to poor hygiene practices adopted during slaughtering, transportation and handling. At all the retail outlets in Thrissur, slaughtered animals were brought from Kuriachira Corporation slaughter house in the early morning. The slaughtering and dressing of the carcass was done on the bare floor of the abattoir which was contaminated with blood, rumen contents and other waste from preceding slaughter and dressing. The carcass was then transported to the shop in trucks at room temperature. Meat outlets, where carcasses hung are also under poor sanitary conditions surrounded by dust and flies. Leftovers were kept frozen and sold on next days. In most of the markets the bench tops are either wooden or tiled and the chopping boards were large blocks of wood. The cleaning procedure followed for tables and equipments were flushing with running tap water, but they were seen with blood stains, accumulated fat and dirt. All these practices expose the meat to contaminants and the risk of being a source of foodborne pathogens including *Listeria* spp.

The prevalence of Listeria in meat and meat products is influenced by many factors including geographical differences, difference in animal rearing, handling and slaughtering practices, and differences in food handling practices including storage conditions. (Johnson *et al.,* 1990)

The occurrence of *Listeria* spp. in the study remains fairly low in comparison with the study of Bouayad and Hamdi, 2012 and Jalali and Abedi, 2008 in Iran whereas the count is higher than that reported by Meyer et al., 2011 (two per cent).

It is generally assumed that raw meat products cannot be free from Listeria because of slaughter methods and food processing that allows greater chance of contamination. During slaughter the main sources of carcass contamination with *L.monocytogenes* are from the colon or its content (Skovgaard and Norrung, 1989), the hide (Takahashi *et al*., 2007) and the tonsils of slaughter animals (Autio *et al*., 2004). The source of Listeria can be the crevices in the bench tops and equipments such as knives or chopping boards.

Retailing meat at ambient temperature will favour rapid growth of this bacterium. Refreezing or chilling will not kill or retard the growth of this as it is a psychrotropic organism. People handling food at different stages can also be a source of contamination. The tradition of consuming under cooked meat or smoked meat exacerbates the public health risk associated with *L.monocytogenes*. Food recontamination might result from contact with equipment and mishandling practices. In addition, contaminated raw meat is also a potential source for cross contamination to heat treated or ready to eat products during processing or in the kitchen (Vitas and Garcia- Jalon, 2004).

The thermal resistance of *Listeria* spp. and *L. monocytogenes* has been debated, but it is generally accepted that proper heating kills viable Listeria cells. According to these results, the existence of *Listeria* spp. in meats would pose a threat only if the meat were insufficiently cooked or if there is chance of cross-contamination. In order to minimize human listeriosis, foods should be cooked to an internal temperature of 70 ^{0}C for more than 20 minutes to ensure destruction of *L.monocytogenes*. Stored cooked food should be reheated thoroughly (70 ^{0}C) to avoid post contamination. Proper cold storage of meat and meat products (freezing -18 ^{0}C) and proper personal hygiene of food handlers is essential to control listeriosis (Mahmood *et al*., 2003).

5.1.2. Chicken

In the present study the prevalence of *Listeria* spp. in chicken was 2.67 per cent out of 150 raw poultry samples collected from retail outlets in Thrissur. Four isolates of *Listeria* spp. were obtained, of which three were *L.monocytogenes* and one was *L.ivanovii.* Jalali and Abedi, 2008 and Peckrovic *et al.,* 2008 could not detect *L. monocytogenes* from raw chicken samples either by conventional method or PCR technique.

The prevalence of *Listeria* spp. in raw chicken meats could be attributed either to fecal contamination during evisceration, or by human handlers (Fenlon *et al.,* 1996). Poultry may also harbour *L.monocytogenes* in their intestinal tract and as such are a potential source of contamination (Capita *et al.,* 2002). The poultry dressing facilities at the retail level are completely manual, with no apparent sanitary measures taken for either the dressing floor or the workers. The bench tops and knives were always blood stained and offals were scattered around with flies. The presence of *L. monocytogenes* in foods could be attributed to contamination from infected animal waste, or caused by chopping board, knives, cleaning cloth, other working surfaces and human contact (Lowry and Tiong, 1985) or unsanitary food production or storage practices followed.

Mena *et al. (*2004*)* recovered *L.monocytogenes* in Spain from 36.1 per cent samples where as Yucel *et al*., 2005 from 21.6 per cent of raw chicken samples sold in Italy, which was much higher than the present study. Several studies showed that the rates of *L. monocytogenes* varied between 23 per cent and 60 per cent in poultry (Pini and Gilbert, 1988; Skovgaard and Morgen, 1988). Comparatively lower prevalence of *Listeria* spp. in chicken may be because the sample was collected immediately after slaughter. In all the shops the poultry was slaughtered and processed in front of the customers since Indian consumers prefer freshly slaughtered birds.

L.ivanovii has been isolated from one raw chicken sample. Though not common, it has been associated with cases of human listeriosis (Guillet *et al.,* 2010).

L. monocytogenes in raw chicken cannot be considered as important as in ready-to-eat products since the raw products are normally cooked before consumption. It has been demonstrated that conventional cooking would eliminate this organism (Norrung, 2000). Several studies indicated that post-processing contamination of ready-to-eat products with *L. monocytogenes* may pose a hazard. So it is obvious that the relevant regulatory agencies need to review the way meat and poultry are sold at local markets. Meat and poultry should not be sold at ambient temperature and sales according to safety procedures. The presence of *L.monocytogenes in* foods might not be extremely hazardous to the "healthy" general public but does pose a serious health hazard to immunocompromised individuals.

5.1.3. Seafoods

The seafoods selected for the present study were prawns and sardines. None of the 30 samples of sardines screened contain *Listeria* spp., whereas the prevalence rate in prawns were 13 per cent and the species identified were *L.monocytogenes* (11 per cent) and *L. grayi* (two per cent).

L. monocytogenes and other *Listeria* species have been isolated from fishery products on a regular basis in tropical regions, since the late 1990s. Embarek (1994) found that the prevalence of *L. monocytogenes* in seafoods varied from four to twelve per cent in surveys from temperate areas. Parihar *et al*. (2008) in a study on isolation of *Listeria* spp. from seafoods reported the prevalence rate of *L.monocytogenes* as nine per cent in Goa and Jemmi and Keuch, 1994 reported it as ten per cent in Switzerland. Jeyasekaran *et al.* (1996) recorded a 13.8 per cent prevalence of *L. monocytogenes* in fish and shellfish in India. All these findings were in agreement with the present study whereas the prevalence of *Listeria* spp. in prawn reported by Dhanashree *et al*, 2003a (9.1per cent) in Mangalore and Gawade *et al,* 2010 (7.69 per cent) in Goa was lower than the findings of the present study.

Jeyasekaran *et al.* (1996) and Dhanashree *et al.* (2003a) reported the prevalence of *Listeria* spp. in sardines as 33.3 per cent where as it could not be detected from sardines sold in Thrissur in the study. Fuchs and Surendran, 1989; Manoj *et al.,* 1991 and Karunasagar and Karunasagar, 2000 reported the absence of *L. monocytogenes* in tropical fish in India. Other studies showed that the prevalence of *L. monocytogenes* in raw fish is quite low, ranging from zero to one per cent (Autio *et al.,* 1999; Johansson, 1999).

The seafoods can be contaminated long before their capture. The *Listeria* spp. can tolerate salt concentration of up to 10 per cent and hence seawater can act as primary source of contamination (Rodas-Suarez, 2006). The contamination may also occur from fish capturing vessels, fish nets, equipments or human handlers and avian sources. The unhygienic way of transportation in ice packs and ice blocks may itself spread the pathogen.

Potential sources of *L. monocytogenes* on fishing vessels include contamination from water and ice, soiled surfaces and boxes, as well as contamination from human and avian sources. Agricultural run-offs as well as sewage or fecal contamination of aquatic bodies can also aid in spreading of the pathogen or association with fish and shellfish (Hansen *et al.,* 2006). Since *L.monocytogenes* is commonly found in coastal waters and in surface waters of lakes, fish captured or cultivated in these waters may possibly carry this microorganism (FAO, 1999). Process contamination in particular has proved to be an important source of Listeria contamination in food production and numerous studies show that in-house *L. monocytogenes* flora contaminates seafood during processing. The incidence of *L. monocytogenes* in raw/fresh seafoods may have serious impact on its processing as it tends to contaminate the processing table as well as frozen seafoods for exports.

Contamination in seafood establishments may occur from any food contact surface as well as from secondary contamination sites and equipment support structures. It is indeed necessary to create awareness among the retailers of the risks that can arise from contamination of foods with *L. monocytogenes* so that appropriate measures can be instituted. Sanitary conditions of food contact surfaces and handling areas, and personal hygienic practices should reduce the potential contamination with *L. monocytogenes* at the retail level.

Isolation of *L. monocytogenes* from seafood suggests that there is a risk of acquiring listeriosis through seafoods in India. *L. monocytogenes* will be killed by cooking and raw or semi-raw seafood (graved or cold-smoked) are not consumed in India. However, *L. monocytogenes* in raw seafoods may pose a health risk in kitchen if cross contaminating cooked other ready-to-eat food. Considering outbreaks of listeriosis associated with different foods, avoidance of consumption of insufficiently cooked seafoods by at-risk populations is recommended. Diligent enforcement of sanitary conditions of food contact surfaces and handling areas, and personal hygiene practices should reduce the potential contamination of fishery products by *L. monocytogenes* at the retail level. Therefore, for public health matter, it was suggested to eviscerate fish immediately after harvesting to avoid bacteria attacking to other tissues. Furthermore, since cross contamination has been considered as a major cause of fish contamination with *Listeria* spp., transportation, handling and processing of fish and fish products should be performed in extreme hygienic condition.

The overall prevalence of *Listeria* spp. was 5.71 per cent in foods of animal origin screened. The highest prevalence was noticed in prawn (13 per cent), followed by beef (4.76 per cent) and chicken (2.67 per cent). The bivalves including prawns are filter feeders. They ingest the organic material that they encounter at the bottom of the sea and accumulate more microorganisms including *Listeria* species and this may be one of the reasons for more prevalence of *Listeria* spp. in prawn (Gawade et al., 2010). The broiler chickens selected for the study were of deskinned which may be the reason for lower contamination rate in chicken.

Strategies to reduce *L. monocytogenes* in foods and consequently listeriosis will depend much on hygienic and sanitary production and processing practices. This is to reduce the colonisation, transmission and cross contamination of *L.monocytogenes* in foods and the environment. An effective control measure for this pathogen has to target the farm, processing plants and the environments. At all these stages, strict adherence to standard operating measures must be practiced. In farming, livestocks should be reared in clean dry environments. Soils in particular should not be moist or damp as that will provide a conducive environment for the growth of this pathogen. Livestock houses should be thoroughly cleaned, and disinfected on a regular basis. Entering of wild animals (which may serve as reservoirs) into the farm should be avoided.

In processing plants, there is the need for each company to set up processing and environmental monitoring plans for *L. monocytogenes*. Such plans must be specified in the HACCP plan of the company. Monitoring plans should lay emphasise on sanitation practices, processing and packaging operations, personnel hygiene, and routine testing programs for *L. monocytogenes*. If the pathogen is found during monitoring, investigations must be carried out immediately to determine the source to prevent further transmission. The management needs to set clear policies and train employees so that they understand the importance of proper sanitary practices. Practices such as moving people and equipment from raw material areas to finished product areas, not wearing clean gloves, handling unsanitary utensils or equipment and then touching finished products should be avoided. Cooling units should have dehumidifying properties in order to limit moisture in this area. Packing materials should also be palletized and covered until used. In retail display, temperatures of refrigerators should be monitored on regular basis, avoid mixing products from different sources, and products should be well packaged for display. Expired products should be disposed off immediately. Further education of consumers on food safety issues is recommended. Also foods should be well cooked or heated (in case of ready-to-eat foods) before being eaten.

5.2. IMMUNOLOGICAL TECHNIQUE

Lateral flow assay was used for the confirmation of *L.monocytogenes* isolates obtained. The 22 isolates of *Listeria* spp. obtained by conventional culture method were subjected to lateral flow assay. Among that 18 were confirmed as *L.monocytogenes* based on carbohydrate utilization test and pathogenicity assay whereas thirteen isolates (59.09 per cent) were confirmed by lateral flow assay.

Some of the samples positive by culture method tested negative with lateral flow assay. This is in agreement with Meyer *et al*., 2011 were majority of *Listeria* spp. positive samples tested negative for *L.monocytogenes* by Vitek Immuno Diagnostic Assay System (VIDAS). They suggested that either the level of background flora was too high, giving a false negative result on culture, or the VIDAS results were positive due to reactions with competitive flora.

Suspected colonies of Listeria on conventional isolation media need to be confirmed to the genus level and then subjected to confirmation tests to identify pathogenic *L. monocytogenes* from other *Listeria* spp. These confirmatory tests tend to be labour-intensive, require a wide variety of media and reagents making confirmation costly, and is time consuming taking several days to complete (Hedge *et al.*, 2007) while immediate action should be taken in case of contamination since it is of fundamental importance to ensure the safety of food. The drastic increase in the foodborne infection caused by *Listeria* spp. demanded reliable and rapid methods for the detection. Apart from the cultural methods, immunological techniques are more popular because of their better specificity and rapid detection efficiency.

The methods for immunological detection fall into four basic categories: visual immunoprecipitation (based on immunochromatography), latex agglutination, immunomagnetic separation and enzyme based immunoassay (Gorski, 2008). All work on the same principle, in which specific anti- Listeria or anti *L.monocytogenes* antisera provided in a matrix bind to antigens on target bacteria. Immuno detection kits are simple to use and give results rapidly.

Lateral flow assay utilize visual immunoprecipitation and give result within 30 min. of sample application. The sample is applied to the chromatography paper and absorbed to the reaction zone containing colloidal, gold labeled antibodies specific to *L.monocytogenes* antigen.

The comparison of the detection of *L.monocytogenes* by lateral flow assay with culture technique, showed that lateral flow assay can be used for routine screening out of Listeria negative samples, the main advantage being a markedly reduced assay time. The false negatives produced may be due to the low number of *L. monocytogenes* present in the samples because of competitive inhibition by other *Listeria* species. Hence the kit cannot be relied upon for detecting *L.monocytogenes* positive samples but can be used to rule out negative sample which is more useful in food industry because of the limited shelf life of the products.

5.3. POLYMERASE CHAIN REACTION

Of the 385 food samples examined, 22 isolates were recovered. All the positive isolates were subjected to PCR for confirmation of *L.monocytogenes*. Of these 18 were *L.monocytogenes* by cultural method where as sixteen (72.73 per cent) were confirmed by PCR.

The findings of the present study is higher than that with Gawade et al, 2010 who confirmed five samples were positive for *L.monocytogenes* by PCR out of 20 Listeria isolates screened. Gouws and Liedemann (2005) obtained a few negative results from PCR when presumptive positive colonies from culture media were used which was in agreement with our study. In contrary to the present study Aznar and Alarcon, 2003 detected *L.monocytogenes* in 56 samples by PCR whereas only 16 were confirmed by culture method.

The wide application of nucleic acid amplification techniques and the increasing industrial interest towards rapid methods has led to the development and application of PCR based methods for the detection of microbial pathogens in food (Germini *et al.,* 2009). PCR techniques are sensitive, highly specific and allow rapid processing times, and they also enhance the likelihood of detecting *Listeria* spp. without the need for isolating pure cultures (Aznar and Alarcon, 2003). Moreover, PCR methods have been applied successfully to the detection and identification of pathogenic organisms in clinical and environmental samples (Kaur *et al.,* 2007).

The PCR amplification relies on two single stranded oligonucleotide primers (usually of 20 to30 bases in length) that flank the front and rear ends of a specific DNA target, a thermostable DNA polymerase that is capable of synthesizing of the specific DNA, and double stranded DNA to function as a template for DNA polymerase. The PCR amplification process usually begins at high temperature (95 ^{0}C) to convert the double stranded template DNA into single strands, followed by a relatively low temperature (55^{0}C) to enable annealing between single stranded primer and single stranded template, and then at 72 ^{0}C to enable DNA polymerase extending along the template. The whole denaturing, annealing and extension process is repeated 25 to30 times so that one copy of DNA template is turned into billions of copies within three to four hours.

The target genes for PCR detection of *L. monocytogenes* are the *hlyA* gene (encoding LLO) (Thimothe *et al.,* 2004), the *iap* gene (encoding an invasion-associated protein) (Schmid *et al.*, 2003), *inlB* (encoding internalin B) (Jung *et al.*, 2003), plcA (encoding phosphatidylinositol phospholipase C), *plc*B (encoding phosphatidylcholine phospholipase C), *actA* (encoding Act A), mpl (encoding metalloprotease), *prf*A (transcriptional regulator Prf A), and *pep*C (aminopeptidase C). Among these genes, the most commonly used has been *hly*A (Aznar and Alarcon, 2002) which was used in this study.

PCR-based methods have several limitations. It may be liable to carry over contamination from the previously amplified products, leading to false positive results. Another notable shortcoming of PCR based assay is its inefficiency in amplifying target of interest directly from food matrix as the fat and proteins in food samples, may interfere with PCR by inhibiting DNA polymerase directly or indirectly by binding Mg^{2+}, as the Mg^{2+} is required for activity of the Taq polymerase (O'Connor, 2003). The matrix-based interference necessitates the use of DNA purification steps that add to both the cost and completion time of the method. Culture enrichment may be required to concentrate the pathogen to improve the sensitivity of detecting the target gene. Analysis on an agarose gel is labour intensive when done on a large scale required by the food processing industry. The PCR only detects the presence of DNA. This does not indicate whether the pathogens are dead or alive (O'Connor, 2003). The food industry must know whether the food represents a health hazard, not whether the pathogen was present at one point but was killed by the food processing method. Some *L.monocytogenes* positive samples go undetected due to overgrowth by other *Listeria* species and/or natural background flora during enrichment and differing abilities of Listeria strains to grow competitively (Ryser *et al.*, 1996). This may explain why negative results were obtained from PCR when using presumptive positive colonies from PALCAM agar.

PCR is deemed to be more reliable than conventional identification since it is based on stable genotypic characteristics rather than relying on biochemical or physiological traits, which can be genetically unstable (Lawrence and Gilmour, 1994). With bacterial adaptation to different environments causing similarities in phenotype, as well as resistance to ingredients in enrichment and selective media, the transition from conventional methods of detection to genetic methods should be carried out. This method proved to be reliable, cost effective and time saving.

5.4. ANTIBIOGRAM OF ISOLATES

In the present investigation on occurrence of *Listeria* spp. in beef, chicken and seafoods, twenty two isolates were obtained in total and 18 of them were *L.monocytogenes*. Antibiogram of the isolates based on Bauer-Kirby disc diffusion assay, revealed that all of the *Listeria* spp. obtained were sensitive to five of the thirteen tested antibiotics. The sensitive antibiotics include erythromycin (15µg), vancomycin (30 µg), doxycycline (30 µg), cotrimoxazole (25 µg) and gentamicin (10 µg). All of the isolates were resistant to ampicillin (10 µg) and cloxacillin (10 µg).

Although the incidence of listeriosis is low, the high mortality rate (up to 30 per cent) requires early diagnosis and appropriate antimicrobial therapy. *Listeria* spp. have been known to be susceptible to many antibiotics that are active against Gram positive bacteria (Hawkins et al., 1984), but more recently, reports of resistance in *Listeria* spp. have been published (Schwaiger *et al.,* 2010.). Since the first report of antibiotic resistance of Listeria strains (Poyart-Salmeron, 1990), there has been a continuing emergence of Listeria strains isolated from food, meat, environment and clinical isolates which are resistant to two or more antibiotics (Arpin *et al.,* 1992; Charpentier *et al.,* 1995).

The study of the antimicrobial sensitivity pattern of *L. monocytogenes* isolates by Bouayad and Hamdi (2012) showed sensitivity to gentamicin (10 mg), tetracycline (30 mg), trimethoprim/sulfamethoxazole (1.25/23.75 mg) and chloramphenicol (30 mg) which was similar to the present work. Mauro *et al.* (2007) reported that the ampicillin, rifampicin, or penicillin plus gentamicin remain the drug of choice for most manifestations of listeriosis and co-trimoxazole as second-choice therapy. Vancomycin and erythromycin are also used, respectively, to treat bacteremia and listeriosis in pregnant women. But the present study was in accordance with the findings except in the case of ampicillin and rifampicin. The resistance to ampicillin by all isolates in this work was in agreement with Pesavento *et a*l, 2010. The resistance pattern shown by *L. monocytogenes* and *Listeria* spp. strain to cloxacillin agreed with earlier work done by Adetunji and Isola (2011).

Recently there are reports of clinical strains resistant to chloramphenicol, erythromycin, streptomycin, tetracycline, vancomycin and trimethoprim (Biavasco *et al*., 1996; Charpentier *et al.,* 1994, 1995). Resistant *L.monocytogenes* strains have also been found in food samples (Roberts *et al.,* 1996). These investigations suggest that *L. monocytogenes* is slowly becoming antibiotic resistant by acquisition of known antibiotic resistance genes from Gram-positive bacteria (Charpentier and Courvalin, 1997).

The cause for resistance against few antibiotics revealed to be the abuse of antibiotic usage in cattle. The public health significance of these findings is that the resistant strains from meat tables may find their way into human population through food chain and occupational exposure. The reason for antibiotic resistance can be explained by the widespread use of antibiotics in food animals as growth promoters and for prophylaxis and treatment of diseases.

While many antibiotic-resistant bacteria in foods are currently saprophytic or commensal in habit, their resistance genes can be transferred, through movable genetic elements such as transposons and plasmids (Poyart-Salmeron *et al.,* 1990), to other food-borne bacteria, including *L. monocytogenes* within the gastrointestinal tract (Perreten *et al.,* 1997). The common sources of resistance genes for *L. monocytogenes* appear to be enterococci and streptococci (Biavasco *et al.,* 1996). However, resistance genes could also come from other sources since such genes often flux among Gram-positive and Gram-negative bacteria through conjugative mobilization (Charpentier and Courvalin, 1999). This process may have undesirable clinical implications for the host and for the wider population coming into contact with derived antibiotic resistant pathogens. Emergence and dissemination of antibiotic resistance in *L. monocytogenes* may have significant future clinical implications for the treatment of listeriosis.

Moreover, evidence of the emergence of multidrug resistant *L. monocytogenes* strains from various sources has been reported (Rodas-Suarez *et al.*, 2006). The results of the present study provide further evidence of the emergence of multidrug resistant strains in nature, representing a potential threat to human health.

The use and misuse of antibiotics contribute to the development of resistance and it is generally agreed that this is a function of the span of time and use; therefore, it is of basic importance to implement monitoring systems. A common limitation of monitoring systems is that they usually consider the resistance to antimicrobial drugs of clinical isolates only. In view of the expected correlation between animal food and human clinical illness, it is estimated to direct investigation towards strains isolated from food also. More restrictions on the irrational use of antibiotics and public awareness activities should be undertaken to alert the public on the risks of the unnecessary use of antibiotics. There should be extensive educational programmes for abattoir workers, meat butchers and buyers for proper hygiene on meat tables and the need for comprehensive HACCP program. Governments at all levels should put in place a nationwide surveillance programme to monitor microbial trends and resistance patterns of pathogens of foodborne origin.

The study warranted the necessity for continuous monitoring and surveillance programme for the occurrence of *Listeria* spp. in all foods of animal origin, fruits, vegetables, environmental samples, feed and fodder and among human and animal patients throughout the state. The study also suggested the need for improved food safety through the implementation of hygienic measures at all levels from production to consumption.

Summary

6. SUMMARY

Listeriosis is an important emerging foodborne bacterial zoonosis. During the last twenty years there has been an increasing concern worldwide about *L.monocytogenes* and its implications on food safety. Several well documented foodborne outbreaks have been described and the organism has been isolated from a wide range of raw and ready-to-eat meats, poultry, dairy products, seafoods, vegetables and from various food processing environments.

In the present study an investigation on occurrence of *Listeria* spp. in beef, chicken and seafoods was carried out. The samples were collected from retail outlets in Mannuthy and Thrissur, local vendors and corporation slaughter house at Kuriachira in Thrissur for a period of nine months from July, 2011 to March, 2012. Hundred gram of the sample was taken from each food item.

For the isolation and identification of *Listeria* spp. from various food samples, the United States Department of Agriculture (USDA) method was used with modifications. Isolation of *Listeria* species from food was carried out by two step enrichment in University of Vermont medium (UVM) I and UVM II followed by selective plating in Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar. The characteristic gray green colonies of Listeria with a black sunken centre and a halo were subjected to various tests and were identified based on the cultural, morphological and biochemical characteristics. Carbohydrate utilization test was used for identification at species level. Then in vitro pathogenicity assay was carried out to confirm the pathogenic species.

The occurrence of *Listeria* spp. in beef, chicken and seafoods were 4.76, 2.67 and 13 per cent respectively. *L.monocytogenes* was detected in four samples of beef, three samples of chicken and eleven samples of prawns. None of the sardines screened were positive for *Listeria* spp. *L. grayi* were detected in two and one samples of prawn and beef respectively. One isolate of *L.ivanovii* was obtained from chicken. The overall prevalence of *Listeria* spp. was 5.71 per cent by conventional culture technique and *L.monocytogenes* was detected in 4.67 per cent of samples.

Lateral flow assay based on immune flow principle was used for the confirmation of *L.monocytogenes* in food samples. Singlepath L ‘mono (Merck), a Gold Labelled Immuno Sorbent Assay (GLISA) was used for qualitative detection and confirmation of the organism. All the isolates of *Listeria* spp. obtained in the study were subjected to Lateral Flow Assay for confirming *L.monocytogenes*. Out of these 22 isolates, 13 were positive for *L.monocytogenes* by lateral flow technique. Evaluation of the lateral flow assay was carried out with culture technique and the kit has a specificity of 100 per cent. The comparison of the detection of *L.monocytogenes* by lateral flow assay with culture technique, showed that lateral flow assay can be used for routine screening out of Listeria negative samples, the main advantage being a markedly reduced assay time.

Polymerase chain reaction targeting listeriolysin O gene (*hly*A) of *L.monocytogenes* was carried out for all isolates of *Listeria* spp. obtained from beef, chicken and seafoods. Out of 22 isolates of Listeria subjected to PCR, sixteen were confirmed as *L.monocytogenes*.

The antibiotic sensitivity pattern of 22 isolates of *Listeria* spp. obtained from three categories of foods of animal origin was carried out. Listeria isolates were tested against 13 antimicrobial agents commonly used in veterinary and human therapy. The antibiotics agents used were ampicillin, amoxycillin, cefotaxime, cefuroxime, chloramphenicol, cloxacillin, cotrimoxazole, doxycycline, erythromycin, gentamicin, rifampicin, streptomycin and vancomycin All the Listeria isolates showed 100 per cent sensitivity to doxycycline, gentamicin, erythromycin, vancomycin and cotrimoxazole. The zone of inhibition was maximum for doxycycline. All the isolates were resistant to ampicillin and cloxacillin.

The study has demonstrated the occurrence of *L.monocytogenes* and other *Listeria* spp. in beef, chicken and seafood. The expanding population of immunocompromised individuals together with the high prevalence of the organism in foods indicates the necessity for establishing appropriate control and preventive measures to reduce risks associated with the occurrence of listeriosis. The study also suggested the need for stringent food safety measures through the implementation of hygienic practices at all levels from the production to the consumption.

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Annexures

ANNEXURE I

Composition of Reagents

1. Kovac’s indole reagent

Composition:	p-Dimethylaminobenzaldehyde	5gm
	Amyl alcohol	75ml
	Hydrochlorc acid (Concentrated)	25 ml

2. Taq DNA Polymerase

The enzyme with a concentration of three unit/ microlitre

3. **PCR reaction buffer E (10X)**

Composition: 100mM Tris (pH 9.0)

500 mM KCl

15 mM $MgCl_2$

1% TritonX-100

Storage: -20 ^{0}C

4. dNTP mix of 10 mM

Composition: dNTP mix is a premixed solution containing sodium salts of dATP, dCTP, dGTP and dTTP, each at 2.5 mM in water

Storage: -20°C

5. $MgCl_2$ of 25 mM

Final concentration of 2mM

6. Forward and reverse primer set

Each of 20 picomole/ microlitre

7. Tris Borate EDTA (TBE) Buffer (10X)

Composition: 89mM Tris base

89mM Boric acid

2mM EDTA

TBE Buffer (1X): The 10X buffer was diluted 10 times with triple distilled water to prepare working 1X TBE buffer.

8. Agarose gel (1 per cent)

One gram of agarose was dissolved by heating in 100 ml 1X TBE buffer.

9. Gel loading buffer

Composition:

Sucrose 40% (w/v)	4g
Bromophenol Blue 0.25% (w/v)	25mg
Xylene cyanol	25mg
Distilled water	10 ml

10**. Ethidium Bromide 10mg/ml (w/v)**

Ten milligrams of ethidium bromide was dissolved in one milliliter of triple distilled water and stored in a dark and cool place at 4^0C in amber coloured bottle.

ANNEXURE II

Composition and reconstitution of media used

1. Listeria Enrichment Medium Base (UVM) (HiMedia)

Ingredients	**Gms/ litre**
Casein enzymic hydrolysate	5.00
Proteose peptone	5.00
Beef extract	5.00
Yeast extract	5.00
Sodium chloride	20.00
Monopotassium dihydrogen phosphate	1.35
Disodium hydrogen phosphate	12.00
Esculin	1.00

Final pH (at 25 ^{0}C) 7.4 ±0.2

Suspend 27.17 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes. Cool to 50 ^{0}C and aseptically add rehydrated contents of one vial of Listeria UVM Supplement I or one vial of Listeria UVM supplement II as per the requirement. Mix well and dispense as desired.

2. Listeria UVM Supplement I (HiMedia)

Formula (per vial, sufficient for 500 ml medium)

Acriflavin	6.00 mg
Nalidixic acid	10.00 mg

Rehydrate the contents of one vial aseptically with 2 ml of sterile distilled water. Mix well and aseptically add to 500 ml of sterile, molten, cooled (45 to 50^0C) Listeria Enrichment Medium Base (UVM)/ Listeria Enrichment Hiveg Medium Base (UVM). Mix well and dispense as desired.

3. Listeria UVM Supplement II (HiMedia)

Formula (per vial, sufficient for 500 ml medium)

Acriflavin	12.50 mg
Nalidixic acid	10.00 mg

Rehydrate the contents of one vial aseptically with 2 ml of sterile distilled water. Mix well and aseptically add to 500 ml of sterile, cooled (45-50 ^{0}C) Listeria Enrichment Medium Base (UVM)/ Listeria Enrichment Hiveg Medium Base (UVM). Mix well and dispense as desired.

4. Listeria Identification Agar Base (PALCAM) (HiMedia)

Ingredients	Gms/ litre
Peptic digest of animal tissue	23.00
Starch	1.00
Sodium chloride	5.00
Mannitol	10.00
Ammonium ferric citrate	0.50
Esculin	0.80
Dextrose	0.50
Lithium chloride	15.00
Phenol red	0.08
Agar	13.00

Final pH (at 25 ^{0}C) 7.0 ±0.2

Suspend 34.5 grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes. Cool to around 50 ^{0}C and aseptically add rehydrated contents of one vial of Listeria selective supplement (PALCAM). Mix well and pour into sterile Petri plates.

5. Listeria Selective Supplement (PALCAM) (HiMedia)

Formula (per vial, sufficient for 500 ml medium)

Polymyxin B sulphate	50,000 IU
Ceftazidime	10.00 mg
Acriflavin hydrochloride	2.50 mg

Rehydrate the contents of one vial aseptically with 5 ml of sterile distilled water. Mix well and aseptically add to 500 ml of sterile, molten, cooled (45 to 50 ^{0}C) Listeria Identification Agar Base (PALCAM)/ Listeria Identification Hiveg Agar Base (PALCAM)/ Listeria Identification Broth Base (PALCAM)/ Listeria Identification Hiveg Broth Base (PALCAM). Mix well and dispense as desired.

6. Trypticase Soya Yeast Extract Agar (HiMedia)

Ingredients	**Gms/ litre**
Casein enzymic hydrolysate	17.00
Papaic digest of soyabean meal	3.00
Sodium chloride	5.00
Dipotassium hydrogen phosphate	2.50
Dextrose	2.50
Yeast extract	6.00
Agar	15.00

Final pH (at 25 ^{0}C) 7.3±0.2

Suspend 51 grams in 1000 ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes.

7. Trypticase Soya Yeast Extract Broth (HiMedia)

Ingredients	**Gms/ litre**
Casein enzymic hydrolysate	17.00
Papaic digest of soyabean meal	3.00
Sodium chloride	5.00
Dipotassium hydrogen phosphate	2.50
Dextrose	2.50
Yeast extract	6.00

Final pH (at 25 ^{0}C) 7.3±0.2

Suspend 36 grams in 1000 ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes.

8. Hugh Leifson Medium (HiMedia)

Ingredients	**Gms/ litre**
Peptic digest of animal tissue	2.00
Sodium chloride	5.00
Dipotassium phosphate	0.30
Glucose	10.00
Bromothymol blue	0.05
Agar	2.00

Final pH (at 25 ^{0}C) 6.8 ± 0.2

Suspend 19.4 grams in 1000 ml distilled water. Heat with frequent stirring. Boil to dissolve the medium completely. Dispense in tubes in duplicate for aerobic and anaerobic fermentations. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes. Cool the tubed medium in an upright position.

9. Listeria Motility Medium (HiMedia)

Ingredients	Gms/ litre
Casein enzymic hydrolysate	20.00
Peptic digest of animal tissue	6.10
Agar	3.50

Final pH (at 25 ^{0}C) 7.3 ± 0.2

Suspend 29.6 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes. Allow the tubed medium to cool in an upright position.

10. Brain Heart Infusion Broth (HiMedia)

Ingredients	**Gms/ litre**
Calf brain, infusion from	200.00
Beef infusion from	250.00
Proteose peptone	10.00
Dextrose	2.00
Sodium chloride	5.00
Disodium phosphate	2.50

Final pH (at 25 ^{0}C) 7.4 ± 0.2

Suspend 37.0 grams in 1000 ml distilled water. Dispense into bottles or tubes as desired. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes. For best results, the medium should be used on the day it is prepared, otherwise, it should be boiled or steamed for a few minutes and then cooled before use.

11. MR- VP Medium (Buffered Glucose Broth) (HiMedia)

Ingredients	Gms/ litre
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00

Final pH (at 25 ^{0}C) 6.9 ± 0.2

Suspend 17.0 grams in 1000 ml distilled water. Distribute in test tubes in 10 ml amounts and sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes.

12. Simmons citrate Agar (HiMedia)

Ingredients	Gms/ litre
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromothymol blue	0.08
Agar	15.00

Final pH (at 25 ^{0}C) 6.8 ± 0.2

Suspend 24.28 grams in 1000 mL distilled water. Boil to dissolve the medium completely. Distribute in tubes or flasks. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes.

13. Urea Agar Base (Christensen) (HiMedia)

Ingredients	**Gms/ litre**
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium chloride	5.00
Disodium phosphate	1.20
Mono potassium phosphate	0.80
Phenol red	0.012
Agar	15.00

Final pH (at 25 ^{0}C) 6.8 ± 0.2

Suspend 24 grams in 950 ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 20 minutes. Cool to 50^{0}C and aseptically add 50 ml of sterile 40 % Urea solution and mix well. Dispense into sterile tubes and allow to set in the slanting position. Do not overheat or reheat the medium as urea decomposes very easily.

14. Phenolphthalene phosphate agar (HiMedia)

Ingredients	**Gms/ litre**
Peptic digest of animal tissue	5.00
Beef extract	3.00
Sodium chloride	5.00
Sodium phenolphthalene phosphate	0.012
Agar	15.00

Final pH (at 25 ^{0}C) 7.4± 0.2

Suspend 28 grams in 1000 ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes.

15. Tryptone Water (HiMedia)

Ingredients	**Gms/ litre**
Casein enzymic hydrolysate	10.00
Sodium chloride	5.00

Final pH (at 25 ^{0}C) 7.5 ± 0.2

Dissolve 15 grams in 1000 ml distilled water. Heat if necessary to boiling to dissolve the medium completely. Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes.

16. Nutrient Gelatin (HiMedia)

Ingredients	Gms/ litre
Peptic digest of animal tissue	5.00
Beef extract	3.00
Gelatin	120.00

Final pH (at 25 ^{0}C) 6.8 ± 0.2

Suspend 128 grams in 1000 ml warm (50^{0}C) distilled water. Heat to dissolve the medium completely. Dispense into test tubes. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes.

17. Andrade Peptone Water (HiMedia)

Ingredients	Gms/ litre
Peptic digest of animal tissue	10.00
Sodium chloride	5.00
Andrade indicator	0.10

Final pH (at 25 ^{0}C) 7.4 ± 0.2

Suspend 15.1 grams in 1000 mL distilled water. Heat if necessary to dissolve the medium completely and dispense in test tubes containing inverted Durham's tubes and sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes. Cool to room temperature and aseptically add sterile stock solution of desired carbohydrate to a final concentration of 0.5 per cent to one per cent (w/v).

18. Mueller Hinton Agar (HiMedia)

Ingredients	**Gms/ litre**
Beef, infusion from	300.00
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00

Final pH (at 25 ^{0}C) 7.3± 0.2

Suspend 38.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 ^{0}C) for 15 minutes. Mix well before pouring.

OCCURRENCE OF *LISTERIA* SPECIES IN BEEF, CHICKEN AND SEAFOODS

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ABSTRACT

An investigation on occurrence of *Listeria* spp. in beef, chicken and seafoods was carried out to determine the prevalence rate of *Listeria* spp. in Thrissur. A total of 385 samples consisting of 105 samples of fresh beef, 150 samples of raw chicken, 100 samples of prawns and 30 sardine samples were collected from retail outlets in and around Thrissur for a period of nine months from July, 2011 to March, 2012. Hundred gram of the sample was taken from each food items.

All the samples were subjected to isolation and identification by USDA method with necessary modification. The method consisted of two step enrichment in UVM I followed by UVM II and then selective plating on PALCAM agar. The species level identification was carried out by carbohydrate utilization test and pathogenicity assay.

Out of the 105 samples of beef screened, five samples were positive for *Listeria* spp. by conventional culture technique and among that four were *L.monocytogenes* and one was *L.grayi.* Four samples out of 150 raw chicken samples examined were positive for *Listeria* spp. and three were identified as *L.monocytogenes* and one as *L.ivanovii*. Hundred samples of prawns were selected for the study. Thirteen isolates of *Listeria* spp. were obtained and eleven were *L.monocytogenes* and two were *L.grayi.* All the sardine samples screened were free of *Listeria* species. The overall prevalence of *Listeria* spp. was 5.71 per cent by conventional culture technique and *L.monocytogenes* was detected in 4.67 per cent of samples.

Lateral flow assay based on immune flow principle was used for the confirmation of *L.monocytogenes* in food samples. Out of the 22 isolates obtained by conventional culture technique, 13 were positive for *L.monocytogenes* by lateral flow assay. The kit can be used for screening out negative samples.

Polymerase chain reaction targeting listeriolysin O gene (*hly*A) of *L.monocytogenes* was carried out for all isolates of *Listeria* spp. obtained from beef, chicken and seafoods. Out of 22 isolates of Listeria organism screened by PCR sixteen were confirmed as *L.monocytogenes*..

The antibiotic sensitivity pattern of 22 isolates of Listeria spp. obtained from three categories of foods of animal origin was carried out. All the *Listeria* spp. showed 100 per cent sensitivity to doxycycline, gentamicin, erythromycin, vancomycin and cotrimoxazole. All the isolates were resistant to ampicillin and cloxacillin.