Discussion and Conclusion

In this study different types of technique like preliminary screening, biochemical confirmative test, molecular detection and pathogenicity tests were applied and the systematic observation was recorded. In this chapter the probable reasons behind the results are discussed and its conclusion is presented.

5.1 Discussion:

Preliminary tests and biochemical tests were conducted so that L. monocytogenes positive samples can be isolated from numerous samples within a short span of time. Alongwith the conventional isolation and identification method other molecular techniques were also carried out for the authentication and detailed examination of genetical co-relation on the basis of variations and similarities using RAPD analysis.

In molecular analysis, PCR analysis was done first for Listeria genus specific gene 16S rRNA because it only amplifies gene of Listeria species. In this way 86 nos. of Listeria isolates were found. But when PCR analysis was conducted on these 86 Listeria isolates through different Listeria specific virulent gene, it was confirmed that 85 isolates are perfectly L. monocytogenes and one isolate belongs to another species of Listeria. Although all 85 isolates were not equally amplified by all genes, but all of them showed positivity with 16S rRNA gene and any other species specific virulent genes. It was found that when the isolates were recovered from mice, each isolate was amplified by all genes. As it was assumed that the isolates might have lost their virulence efficiency due to stoppage of expression of virulent genes in in-vitro condition. The vitality will be resumed automatically in in-vivo conditions. These results are in accordance with the Roche et al., (2005).

Present study reveals that the prevalence of L. monocytogenes was maximum in cattle (8.3%) in North Eastern zone of India. It was also found that on screening animal and environmental samples, maximum number of L. monocytogenes was isolated from utensils (18.8%) of retail meat seller and the water (27.2%) used for washing them. The
findings contradict with the results obtained in the study conducted by Nightingale et al., 2004, which suggests that the overall prevalence of *L. monocytogenes* is highest in goat population. Analysis of animal samples of the farm supplying meat to the seller resulted in finding of less *L. monocytogenes* prevalence (5.4%) than in the retail meat shops (7.8%). It can be attributed to the source of supply of beef which was from retail meat sellers suggestive of passing of meat through various channels of transport and storage till reaching meat shop, which can result in external/ environmental contamination of *L. monocytogenes* as the organism is ubiquitous in nature. It also can presume that much contamination takes place after reaching the meats in shop as the meats in shops were processed in the same utensils and water from the same containers that are not hygienically clean. If one of the animals was infected with *L. monocytogenes* than this pathogen can spread out in meat table or floor through water and the utensils. Also the studies conducted by Ali et al., 2010; Fratamico et al., 2005; Hedberg et al., 1992 concluded that the meat shops play a significant role in *L. monocytogenes* contamination.

In the present Study, a less percentage (5.4%) of *L. monocytogenes* was observed in screening of the animal samples like nasal swabs, vaginal swabs, blood and faeces obtained from farm animals as compared to those obtained from milk samples (8.77%) collected from the same animal sources of same farm. The results are in resemblance with those obtained in the experiment carried out by Bemrah et al., 1998 which can be attributed to the regular treatment and vaccination of of farm animals. Although, the percentage of positivity in milk samples was comparatively higher which may be due to the reason that *L. monocytogenes* can survive for longer time in the farm water and fecal matter that comes in contact with milk through unclean udders, equipments, human hand, and teats of cattle during milking, that agree with the studies conducted by Jami et al., 2010; Kasalica et al., 2011. On the other side the occurrence of *L. monocytogenes* was higher in domestic animal milk samples (9%) than those of farm animal milk samples (7.6%) may be due to lack of proper treatment of domestic animals.

The isolation protocol standardized by Peggy et al., (1986) using cold enrichment method was followed for isolation of *L. monocytogenes* from milk. The result showed almost five-fold more (8.7%) isolation as compared to the same samples processed by without cold enrichment method (1.7%). According to Peggy et al., (1986) in cold enrichment protocol, most of the bacteria except *L. monocytogenes* present in milk cannot survive in 4°C, which results in higher percentage of isolation of *L. monocytogenes*. 
Discussion and Conclusion...

Though *L. monocytogenes* was found in low quantity in soil, it was found that its prevalence was more in the soil where animal faeces had been used as organic fertilizer. According to Dowe et al., (1997), it was realized that through the faeces of infected animal *L. monocytogenes* comes in the soil that contaminates the crops. Subsequently it infects human being through animals. So, it is evident that herbivorous animals serve as media of *L. monocytogenes* that agrees with Schlech et al., (1983); Chukwu et al., (2004); Nicholas et al., (2000).

Results obtained in present research, showed that the occurrence of *L. monocytogenes* was higher in Assam (6.96%) followed by Meghalaya (6.19%) and Tripura (5.64%). As the average annual temperature of Assam and Tripura was identical whereas that of Meghalaya was low, also the average humidity in these three states was almost same but rainfall was different. The difference of rainfall may be the key reason of minor difference between *L. monocytogenes* prevalence in these states. The results are in accordance with those obtained in the study carried out by Soyutemiz et al., (2005) suggesting the particular intensity of rainfall causes greater *L. monocytogenes* prevalence in Assam.

In case of samples collected from Mizoram and Manipur there was no isolation of *L. monocytogenes* found. These results obtained can be justified in the light of the source of these samples which was basically from farm animals where the regular vaccination and other hygienic managerial practices are carried out. Consequently, there are very low chances of getting the sample from infected animal.

Along with conventional isolation, identification and molecular techniques for *L. monocytogenes*, the collection of meteorological data was also done. This data was particularly collected for the period of sample collection irrespective of the geographical location. The meteorological data shows excellent co-relation with the isolation of *L. monocytogenes*. It was found that *L. monocytogenes* prevalence was greatly influenced by temperature, humidity and rainfall. This prevalence was found to be increased during summer when there was an increase in temperature, humidity and rainfall. It starts increasing from March and reaches its culmination in June and July. On the other hand, this prevalence decreased from September to February when temperature, humidity and rainfall remain lower. The results are in accordance with the studies of Farber et al., (1989) and Bayles et al., (1996).
Discussion and Conclusion...

Inspite of the excellent correlation between isolation and meterological data, present study shows slight deviation from it. As the isolation done in January from Assam samples was at slightly higher side as compared to the other isolation obtained in accordance of the correlation. This can be attributed to the type of sample processed which was wash water of retail meat shop where the external contamination plays a crucial role in showing the false prevalence.

Molecular detection techniques were applied with the objective of finding virulence characteristics of isolates and also the genetical similarities and differences. Eight serovars were observed through PCR serotyping. viz. 1/2a (23.5%), 1/2b (14.11), 4b (20%), 4d (3.52), 4e (3.52%), 3a (15.29%), 3b (16.47%) and 7 (3.52%). Though there are 13 known serovar, only three of these like 1/2a, 1/2b and 4b are mostly responsible for listeriosis cases. According to Dounith et al., (2004) 95% L. monocytogenes isolates found in food and animal belong to 1/2a, 1/2b, 1/2c and 4b. Though serovar 1/2a is mostly isolated from contaminated food, it was found that serovar 4b causes maximum epidemic listeriosis (Tappero et al., 1995). Kalekar et al., (2011) also recovered serovars 1/2b, 3b, 4b, 4d and 4e by multiples PCR serotyping method from humans in India during 2006-2009. So it can be convinced through PCR serotyping that L. monocytogenes found in North Eastern states are also gravely harmful and may cause greater listeriosis outbreak at any time.

Side by side, it was also found that there was no much difference of serovar types among the isolates of different states. This serovar types were nominally differed on the types of sample source. Most of the isolates found in animals belong to serovar 1/2a, 1/2b, 4b, 3b and 3a while isolates found from environmental source belong to serovar 4d, 4c and 7.

RAPD technique was used to determine the similarity and dissimilarities of DNA sequence between isolates collected from different source. OPA10 made different RAPD pattern for the isolates LM58 from other isolates which was an environmental sample. LM53 an environmental sample that generated totally different RAPD pattern from other isolates by OPA9 primer. Environmental isolates LM60 and LM4 generated RAPD pattern by OPB7 primer that are distinctly different from the other isolates. RAPD pattern of LM40 generated by OPA13 primer isolated from beef was also totally different from others. The RAPD patterns generated by OPA18 primer of LM45 and LM11 isolated from pig were similar but the rest are totally different from each others. Thus the study results
revealed that the isolate of same source mostly makes same RAPD pattern. In the present research study, it was also observed that among the isolates found from the similar type of sources, the serotopic similarity was maximum. Likewise genetic similarity among those isolates of same serovar was also maximum, showing alike amplification of similar cluster of virulent genes that agrees with Bibb et al., 1990 and Wagner et al., 1996.

Ideal antibiotic therapy is based on determination of the etiological agent and its relevant antibiotic sensitivity. Considering that L. monocytogenes is slowly becoming antibiotic resistant by acquisition of known antibiotic resistance genes from gram-positive bacteria, a continued surveillance of emerging antimicrobial resistance of this pathogen is important to ensure effective treatment of listeriosis. In present study, the antibiogram analysis was also undertaken to verify the sensitivity pattern of the recovered isolates of L. monocytogenes. The ampicillin (96.5%) was found to be the most sensitive among all antibiotics used for testing followed by Chloramphenicol (95.3%), Gentamycin (94.1%), Amoxicillin (92.9%) and Cefuroxime (91.8%). The results are in resemblance with those obtained in the experiment conducted by Ruiz-Bolivar et al., (2011) who observed cent-per-cent susceptibility against Ampicillin and 98% susceptibility against Chloramphenicol. Zade and Karpe, (2010) also reported maximum sensitivity of listerial isolates towards Ampicillin (88%) and Gentamycin (77%). The results observed are also matches with Ennaji Hayat et al., (2008) who stated chloramphenicol and Gentamycin as the most effective antibiotics.

In mouse lethality test, it was found that mice injected with isolates showing positive results to all virulent genes in PCR assay died in less time (36 to 48hrs). On the other hand, the isolates not showing positive results to all virulent genes took longer time (4 to 5 days) to kill mice. Interestingly, when again these isolates are recovered from dead mice and analysed by PCR, it was found that all the isolates were showing amplification for almost all virulent genes, which were not expressed before in-vivo revival. When mouse lethality test was repeated with the recovered isolates, all the mice died almost at the same time like those who were initially showing amplification for all virulent genes (36 to 48 hrs). In accordance with Swaminath et al., (1989) the reason behind it might be that the virulence efficiency of the isolates was dormant in in vitro condition that was regained when it was injected into mice.
5.2 Conclusion:

Listeriosis is a serious invasive bacterial zoonotic disease caused by pathogenic strains of the genus *Listeria*, of which *L. monocytogenes* is the major pathogenic species in both animals and humans. Epidemiological study of *Listeria* in animals and environment revealed that *L. monocytogenes* is largely present in animals and environment of North Eastern Hilly region.

A total of 1550 samples comprising 1237 animal and 313 environment samples were collected from diverse commercial shops, animal farms, and natural sources located in 6 states (Assam, Tripura, Meghalaya, Nagaland, and Mizoram) of NEH region of India in the year 2008 to 2010. The present study indicates that the listerial contamination prevails in the retail meat shops, animals their products and environment of this region, as screening of 1550 samples resulted into 85 isolates of *L. monocytogenes*. The occurrence of virulence genes in most of the isolates revealed that the isolates were equally virulent and pathogenic *L. monocytogenes* as found all over the world, posing great health hazard to the public as well as animals in this region. There seems to be lack of mass awareness in the people, especially those involved in the food production and processing in the region about the importance of personal hygienic habits and practices. Moreover, a large number of abortions take place, both in animals and human, many people suffer from meningoencephalitis; some of these may be due to listeriosis that can only be confirmed after availability and use of suitable diagnostic procedures for this emerging food borne pathogen in the region. Due to lack of awareness and diagnostic facilities, reporting of deadly listeriosis may remain under reported. Hence there is a scarcity of literature available about occurrence of listeriosis in this part of the country.

A correlation was also observed between the isolation in present study with the meteorological data collected from the respective locations. More number of isolates was recovered in the summer season during which the parameters like temperature, humidity and rainfall suggested by the meteorological data were ideal for the growth and multiplication of listerial organism.

Alongwith the conventional isolation and identification, molecular confirmation and characterization of recovered isolates were also carried out to get the idea about their pathogenic nature and genetical similarities and variations within the isolates. PCR serotyping and studies on virulence genes present in the isolates indicated the similarities
in the isolates from same source. High genetic similarity was observed among isolates of same serovar.

Development of multidrug resistance in bacteria is one of the serious problems faced by the health professionals throughout the world. In order to get idea about the status of this phenomenon in listerial isolates, antibiogram study was carried out which revealed the comparatively higher sensitivity of the isolates to Ampicillin and Chloramphenicol. However, some of the isolates also showed multiple drug resistance to some of the studied antibiotics.

Findings on 5.48% isolation rate of *L. monocytogenes* with high pathogenic potential in the samples from different sources during the present investigation indicates the importance of this important pathogen in the region, which is a matter of great concern from public health point of view. Also the results of this study will alert people about nagging public health hazard of listeriosis, providing basis for further detailed epidemiological investigation of this pathogenic organism for the researchers and health professionals.