Internet Journal of Food Safety, Vol.9, 2007, p. 56-62

A Multiplex PCR for Detection of Virulence Associated Genes in *Listeria monocytogenes*

Rawool, D. B.[1]*, Malik, S. V. S.[1], Barbuddhe, S. B.[2], Shakuntala, I.[1] and Aurora, R.[3]

[1]*Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar 243 122 India*
[2]*ICAR Research Complex for Goa, Ela, Old Goa 403 402, India*
[3]*Dayalbagh Educational Institute (Deemed University), Dayalbagh, Agra 282005, India.*

Abstract: Listeriosis is an important food-borne infection caused by *Listeria monocytogenes.* A multiplex PCR assay was developed for detection of four virulence-associated genes of *L. monocytogenes* namely, *plcA*, *hlyA*, *actA* and *iap.* The method was used to detect *L. monocytogenes* in milk samples with and without enrichment and simultaneously comparing it with conventional cultural method. The assay could detect as low as 15 cells after enrichment. Later, the multiplex PCR was successfully employed to detect virulence-associated genes in *L monocytogenes* isolated from various sources.

Key words: Milk, Listeria monocytogenes; multiplex PCR; virulence –associated genes

* Corresponding author. mailing address: Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar 243 122 India, E-mail: drrawool@rediffmail.com

Introduction

Listeriosis is an important food-borne infection caused by *Listeria monocytogenes*. *L. monocytogenes* has been isolated from various foodstuffs including milk (Barbuddhe *et al.,* 2002). Identification of listeriae from samples contaminated with multiple species relies on selective enrichments and subsequent biochemical analyses. It is laborious and requires at least five days for confirmation. *In vitro* amplification of specific DNA sequences by PCR allows direct detection and identification of the pathogen (Herman *et al.* 1995). Multiple key virulence factors such as hemolysin (*hlyA*), phosphatidylinositol phospholipase C (*plcA*), actin polymerization protein (*actA*) and invasive associated protein (*iap*) are important in *L. monocytogenes* pathogenesis (Furrer *et al.,* 1991, Portnoy *et al.,* 1992). Therefore, detection of just one virulence-associated gene by PCR is not always sufficient to identify *L. monocytogenes* (Nishibori *et al.,* 1995). In addition, it is plausible that some *L. monocytogenes* strain may lack one or more virulence determinants because of spontaneous mutations (Cooray *et al.,* 1994). Therefore, simultaneous detection of virulent genes in a single step will be desirable as it reduces time, labour and it will be useful in a large scale survey for detecting virulent strain of *Listeria*.

Direct detection of pathogens from foods by PCR may be problematic due to the presence of a PCR-inhibitory factor in the food (Fluit *et al.,* 1993). Various sample preparation methods have been developed to remove or to reduce the effects of PCR inhibitors in food stuffs (Lantz *et al.* 1994). A pre-enrichment method (Niederhauser *et al.,* 1992) has been reported to overcome the problem of inhibitors. In the present study, we tried to find a procedure enabling the detection of individual virulence-associated genes of *L. monocytogenes* by PCR in milk samples with and without enrichment and simultaneously comparing it with conventional cultural method. Later, we developed a multiplex PCR for simultaneous detection of four virulence-associated genes of *L. monocytogenes* namely, *plcA*, *hlyA*, *actA* and *iap* and employed for PCR analysis of virulence-associated genes in *L. monocytogenes* isolated from various sources.

Materials and Methods

Bacteria. The strains of *L monocytogenes* 4b (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135), *Streptococcus faecalis* (MTCC 439), Bacillus cereus (MTCC 1272), *Escherichia coli* (MTCC 443), *Aeromonas hydrophila* (MTCC 646) used in the study were obtained from Institute of Microbial Technology, Chandigarh, India. The reference strains of *Listeria* namely, *L. monocytogenes* 4b (NCTC 11994), *L. monocytogenes* 1/2a (NCTC 7973), *L. monocytogenes* 1/2b (NCTC 10887), *L.ivanovii* (NCTC 11846), *L. innocua* (NCTC 11288), *L. seeligeri* (NCTC 11856), *L. grayi* (NCTC 10812), *L. welshimeri* (NCTC 11857) were kindly provided by Prof. K.L. Morgan, University of Liverpool, U.K. The strains of *Salmonella* (1117) and *Vibrio cholerae* (0139) were procured from Division of Veterinary Public Health, Indian Veterinary Research Institute, India. The strains were tested for their purity besides morphological and biochemical characteristics.

Polymerase chain reaction. For standardization of PCR a virulent strain of *L. monocytogenes* MTCC 1143 was grown overnight in Brain Heart Infusion broth (BHI) at 37°C. The DNA was extracted as per the methodology of Makino *et al*., (1995). The obtained culture (approximately 1 ml) was centrifuged in a microcentrifuge (Sigma, USA) at 6000 rpm for 10 min. The recovered pellet was resuspended in 100 µl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA), heated in a boiling water bath for 10 min. and then snap chilled in crushed ice. The obtained lysate (5µl) was used as a DNA template in PCR reaction mixture. Bacterial DNA was also extracted employing DNA extraction kit (Mini Prep, Sigma).

PCR was standardized for the detection of individual virulence associated genes namely, *plcA*, *hlyA*, *actA* and *iap* using primer pairs of 5'-CTG CTT GAG CGT TCA TGT CTC ATC CCC C-3' and 5'-ATG GGT TTC ACT CTC CTT CTA C-3' specific for *plcA* (Notermans *et al.* 1991), 5'-GCA GTT GCA AGC GCT TGG AGT GAA-3' and 5'-GCA ACG TAT CCT CCA GAG TGA TCG-3' for *hlyA* (Paziak-Domanska *et al.* 1999), 5'-CGC CGC GGA AAT TAA AAA AAG A-3' and 5'- ACG AAG GAA CCG GGC TGC TAG - 3' for *actA* (Suarez and Vazquez-Boland 2001) and 5'-ACA AGC TGC ACC TGT TGC AG-3' and 5'-TGA CAG CGT GTG TAG TAG CA-3' for *iap* (Furrer *et al.* 1991) of *L. monocytogenes* by optimizing the different conditions that affect the sensitivity and specificity of the reaction.

Based on optimization trials, the standardized PCR protocol for 50 µl reaction mixture included 10 X PCR buffer (100 mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 0.2 mM dNTP mix, 2 mM $MgCl_2$ and 10 µM of a primer set containing forward and reverse primers (a final concentration of 0.1 µM of each primer), 1 unit of Taq DNA polymerase, 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume. The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 2 min followed by 35 cycles each of 15 s denaturation at 95°C, 30 s annealing at 60°C and 1 min 30 s extension at 72°C, followed by a final extension of 10 min at 72°C and hold at 4°C. All the four set of primers for virulence-associated genes were amplified under the similar PCR conditions and amplification cycles. The resultant PCR products were further analyzed by agarose gel electrophoresis (1.5%; low melting temperature agarose L); stained with ethidium bromide and visualized by a UV trans-illuminator (UVP Gel Seq Software, England).

Sensitivity of PCR. The standardized PCR was assessed for its sensitivity. Briefly, *L. monocytogenes* 4b (MTCC 1143) culture grown overnight in BHI broth was centrifuged at 6000 rpm for 10 min. and the pellet obtained was washed once with phosphate buffered saline (PBS), pH 7.2. The concentration of cells was adjusted to 3 x 10^8/ml using McFarland's nephelometric tube No.1 and 10-fold serial dilutions were made upto 3 cell/ml of PBS. The template DNA was prepared by subjecting 1ml aliquot of each dilution to snap-chill method as described above.

Specificity of PCR. The specificity of the standardized PCR was tested by screening the standard strains of *L. monocytogenes*, *Listeria* species as well as some other commonly prevalent and cross-reacting bacterial species (Table 1) with the primers used in this study. The DNA template preparation from the test organisms and other PCR conditions were similar to those described earlier.

Multiplex PCR. The multiplex PCR was standardized for detecting four virulence associated genes of *L. monocytogenes* namely, *plcA*, *hlyA*, *actA* and *iap* in a single reaction tube containing all the four primer sets for these genes. The DNA template preparation from pathogenic strain of *L. monocytogenes,* PCR reaction and agarose gel electrophoresis of the PCR products were done as per the methods employed for the detection of virulence genes of *L. monocytogenes* described earlier. The standardized PCR protocol for 50 µl reaction mixture included 10 X PCR buffer (100mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 1 mM dNTP mix, 6 mM $MgCl_2$ and 10µM of four primer sets (containing forward and reverse primers a final concentration of 0.1 µM of each primer), 4 units of Taq DNA polymerase, 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume. The cycling conditions for multiplex PCR

Table 1. Standard bacterial strains used to check the specificity of PCR

Description of the organism	Virulence genes detected			
	hlyA	*plcA*	*actA*	*iap*
L. monocytogenes 4b (MTCC 1143)	+	+	+	+
L. monocytogenes 4b (NCTC 11994)	+	+	+	+
L. monocytogenes 1/2a (NCTC 7973)	+	+	+	+
L. monocytogenes 1/2b (NCTC 10887)	+	+	+	+
L. ivanovii (NCTC 11846)	-	+	+	-
L. welshimeri (NCTC 11857)	-	-	-	-
L. innocua (NCTC 11288)	-	-	-	-
L. grayi (NCTC 10812)	-	-	-	-
L. seeligeri (NCTC 11856)	+	-	-	-
Escherichia coli (0157H7)	-	-	-	-
Vibrio cholerae (0139)	-	-	-	-
Salmonella (1117)	-	-	-	-
Bacillus cereus (MTCC 1272)	-	-	-	-
Streptococcus faecalis (MTCC 439)	-	-	-	-
Escherichia coli (MTCC 443)	-	-	-	-
Staphylococcus aureus (MTCC 1145)	-	-	-	-
Rhodococcus equi (MTCC 1135)	-	-	-	-
Aeromonas hydrophila (MTCC 646)	-	-	-	-

were similar as described earlier for the detection of individual virulence gene of *L. monocytogenes*.

Artificially contaminated milk samples. Using the above-mentioned PCR setting we tried to detect L. monocytogenes suspended at serial concentrations ranging from 3 x 10 to 3 × 10^8 cells ml^{-1} in commercially available pasteurized cow milk.

The pasteurized milk samples those turned out to be culturally negative for *Listeria* were pooled and stored at –20˚C till used for experimental work. Aliquots (3 ml) drawn from the pooled milk sample were spiked with standard strain of the pathogen and processed for (with and without enrichment) detection of the pathogen by cultural method and PCR. Briefly, milk aliquots (3 ml) drawn from the pooled milk sample were inoculated with the different concentration of test strain ranging from 30 to 3 x 10^8 cells/ml. Each dilution of the sample was distributed in three test tubes, 1ml/test tube designated as set 1, set 2 and set 3. Accordingly 9 test tubes including negative control were included in each set. Of these, two sets not subjected to enrichment (set1 and 2) were analyzed immediately for the detection of the test strain, i.e. one (set1) by the PCR targeted at virulence-associated genes of *L. monocytogenes* and another (set 2) by cultural method. The remaining set (set 3) was enriched in University of Vermont medium 1 (UVM-1) in the ratio of 1: 10 (i.e. 1 part of milk and 9 parts of UVM-1 broth) and incubated overnight at 37°C before attempting detection of the test strain by PCR as well as cultural method. Aliquots (1 ml) from each dilution of the spiked milk samples in set 1 were centrifuged at 12000 rpm for 10 min. The pellet obtained was washed once with PBS (1ml) and recovered by centrifugation at 12000 rpm for 10 min. The washed pellet was dissolved in sterilized milliQ water (100µl), subjected to vigorous heating in boiling water bath for 10 minutes and then snap-chilled in crushed ice for 2 min. The cell lysate was centrifuged at 3000 rpm for 10 min and the supernatant was used as DNA template for detection of the test strain by PCR. Simultaneously, 1 ml of each dilution of the spiked samples in set 2 was spreaded on Dominguez-Rodriguez isolation agar (Dominguez-Rodriguez et al. 1984) and incubated at 37°C for 24 h. Similar procedure was adopted for detection of *L. monocytogenes* suspended in milk after enrichment in UVM-1 by PCR as well as cultural method.

Listeria strains. Twenty strains of *L. monocytogenes* isolated from milk (10), buffaloes with reproductive disorders (5) and fresh water fishes (5) were analysed for the presence of virulence associated genes employing the multiplex PCR.

Results and Discussion

Rapid isolation and confirmation methods for *L. monocytogenes* in foods are still being sought (Beumer and Hazeleger, 2003). But there are certain strains of *L. monocytogenes* which behave phenotypically quite typical and inconspicuous but are non-pathogenic (Hof and

Rocourt, 1992). Thus, to address the pathogenic potential of *Listeria* isolates, *in vivo* methods namely, chick embryo and mouse inoculation tests remain the most reliable and mandatory approach in order to link these isolates with the cases of listeriosis. However, the *in vivo* methods remain objectionable from ethical point of view and need skilled personnel to perform. In view of this situation, it has been suggested that diagnosis of pathogenic *Listeria* spp. and listeric infection should ideally be based on virulence markers (Notermans *et al*., 1991). Moreover, the importance of PCR has been investigated for detection of *L. monocytogenes* from foods (Gouws and Liedemann, 2005). Thus, in the present study we first attempted to standardize a PCR protocol, which could detect individual virulent associated genes (*plcA*, *hlyA*, *actA* and *iap*) of *L. monocytogenes* in milk samples with and without enrichment and simultaneously comparing it with conventional cultural method. Later, we developed a multiplex PCR for simultaneous detection of all the virulence-associated genes of *L. monocytogenes* under study for rapid detection of virulent strains of *L. monocytogenes*.

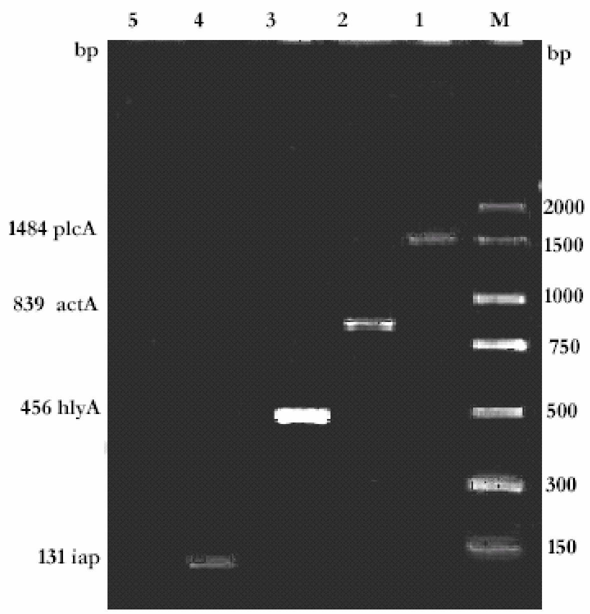

Figure 1. Virulence associated genes of standard *Listeria monocytogenes* (MTCC 1143). M: DNA Marker (150 to 2000 bp), Lane 1: *plcA* gene, Lane 2: *actA* gene, Lane 3: *hlyA* gene, Lane 4: *iap* gene, Lane 5: Negative control.

In the present study the primer sets for all the four genes allowed amplification of 1484 bp (*plcA*), 839 bp (*actA*), 456 bp (*hlyA*) and 131 bp (*iap*) PCR products, respectively, each represented by a single band in the corresponding region of the DNA marker (Fig. 1). These findings commensurate with the published work for detection of *hly*A gene (Paziak-Domanska *et al*., 1999), *plc*A and *prf*A genes (Notermans *et al*., 1991b), *iap* gene (Furrer *et al*., 1991) and *act*A gene (Suarez and Vazquez-Boland, 2001) with respective sets of primers giving no cross-reactions with other bacteria.

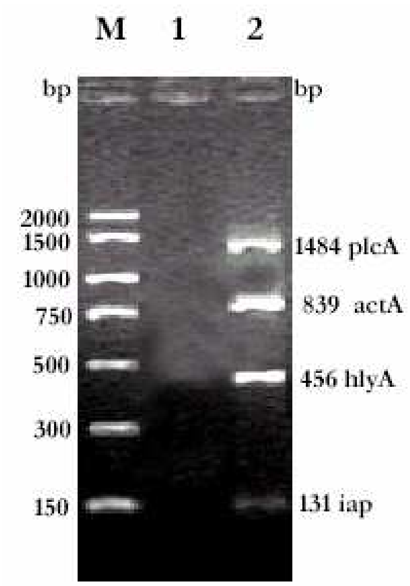

Figure 2. Multiplex PCR of four virulence associated genes for standard *Listeria monocytogenes* (MTCC 1143). M: DNA Marker (150 to 2000 bp), Lane 1: Negative control, Lane 2: Amplified products of four genes (*plcA*, *actA*, *hlyA* and *iap*).

Each of the primer was found to be specific to the target gene as it specifically amplified the PCR product of that gene. All the four genes were detected in standard strains of *L. monocytogenes*, two genes namely, *plcA*, and *actA* in the standard strain of *L. ivanovii* and the *hlyA* gene in the standard strain of *L. seeligeri* whereas, none of the genes was detected in the cultures of the remaining *Listeria* spp. and other bacterial species cultures (Table 1). The amplification of *plc*A gene in *L. ivanovii* can be explained based on the reports that *plc*A and *plc*B genes are present in *L. monocytogenes* and *L. ivanovii* (Vazquez-Boland *et al.*, 2001). The *act*A gene from *L. ivanovii*, *i-act*A, encodes a protein larger than *act*A from *L. monocytogenes*. The similarity between the two proteins is also limited (34% identity), however, *act*A and *i-act*A have a similar overall structure (Gerstel *et al.*, 1996). The amplification of haemolysin gene (*hly* A) in *L. seeligeri* genome could be explained on the basis of homology at both the protein (86-91%) and in the nucleotide (76-78%) of gene sequence of LLO, ILO and seeligerilysin O (LSO) (Mengaud *et al.*, 1988; Haas *et al.*, 1992). However, our amplification system does not yield DNA fragments of *hly*A gene in standard strain of *L. ivanovii*, which can be explained on the basis of report that the genetic information for haemolysin production in *L. ivanovii* could be divergent from *L. monocytogenes* hemolysin gene despite high homology in partial amino acid sequence analysis (Kreft *et al.,* 1989).

Enrichment of food samples prior to PCR analysis overcomes most of the problems and has been recommended by several workers (Wang *et al.,* 1992, Boer and Beumer 1999, Olsen 2000). In the present study, the sensitivity of PCR was compared for the detection of the pathogen in spiked milk samples before and after their enrichment in UVM-1 at 37˚C for 24 h. It was possible to detect as low as $3x10^6$ cells/ml suspended in PBS by using each of the primer set. Compared with the detection limit for bacteria suspended in PBS, the enrichment of milk samples in UVM-1 at 37°C for 24 h has increased the sensitivity of PCR from 3×10^6 cells/ml (before enrichment) to 3×10^3 cells/ml. With the PCR, setting fewer than 15 cells could be detected after enrichment. Similar type of increase in the sensitivity of cultural method was also observed following 24 h enrichment from 3×10^3 cells/ml (before enrichment) to 30 cells/ml of the milk samples in UVM-1. The poor sensitivity of PCR as compared to culture method might be because only 5.0µl of the DNA was used in PCR reaction whereas 1ml of sample (200 times more) was used for plating in cultural method. Our results are in agreement with Cartyyvels *et al.,* (1996) who observed similar results of poor sensitivity of PCR for detection of *Mycobacterium tuberculosis*. They explained that greater sensitivity of the culture method could be due to 40-times greater sample volume used in the culture method.

In the present study, the better detection of the pathogen (as less as 3×10^3 cells/ml) by PCR in the enriched spiked samples compared to the detection level (3×10^6 cells/ml) in samples not subjected to enrichment in UVM-1 might be attributed to the significant increase in the number of the pathogen following enrichment of the samples compared to the relatively lesser number of *L. monocytogenes* present in the milk samples before enrichment. This approach is in agreement with reported detection of *L. monocytogenes* by the PCR in artificially contaminated milk samples (Cooray *et al.,* 1994). Since only 5 µl of lysate was taken as DNA template into the PCR mixture, it indicated that fewer than 15 cells could be detected after enrichment. The present level of sensitivity in the detection appeared to be applicable to the practical survey of milk and milk products for *L. monocytogenes.*

This assay may prove useful for rapid detection of *L. monocytogenes* in milk and milk products and can be adopted for meat and clinical samples. In the food industry the contamination of surfaces, equipment, and food with non-pathogenic *Listeria* spp. is common. Therefore, the ability to rapidly detect pathogenic *Listeria* strains without cross-reactions with non-pathogenic and related strains can potentially improve upon current approaches.

In the present study *L. monocytogenes* strains isolated from various sources were analyzed for the presence of virulence-associated genes employing the multiplex PCR. Different combinations of genes were detected in different isolates (data not shown). The choice of the target gene is of utmost importance for detection of virulent strains of *L. monocytogenes* by PCR. The use of primers specific for *plcA*, *hlyA*, *actA* and *iap* seems to be reasonable and unique to pathogenic *Listeria* (Furrer *et al.,* 1991, Notermans *et al.* 1991, Paziak-Domanska *et al.,* 1999, Suarez and Vazquez-Boland 2001). However, it is plausible that some *L. monocytogenes* strain may lack one or more virulence determinants because of some mutation (Cooray *et al.,* 1994). Moreover, our previous studies have also revealed that using PCR assay for detection of single virulence associated gene is neither sufficient to identify the *L. monocytogenes* isolates nor to reveal its true pathogenic potential as majority of *L. monocytogenes* isolates showed different gene profiles (Rawool *et al.,* 2007; Shakuntala *et al.,* 2006). Therefore, simultaneous detection of virulent genes namely *plcA*, *hlyA*, besides *actA* and *iap* genes in a single step will be desirable as it reduces time, labour and also it would be useful in a large scale survey aiming at detection of virulent strain of *Listeria*.

Summary

The multiplex PCR was successfully employed for the detection of different genes in *L. monocytogenes.* The PCR method reported here could be completed in 6 h without enrichment however since the detection level was poor without enrichment, the authors would like to recommend the use of this multiplex PCR after enrichment for a large scale survey to detect of virulent strains of *Listeria* from milk samples.

Acknowledgements

We thank Director, Indian Veterinary Research Institute, Izatnagar, India for providing facilities for the research work. Thanks are due to Dr. A.R. Datta, US FDA for critically checking the manuscript

References

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

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

Boer, B.D. and Beumer, R.R. (1999) Methodology for detection and typing of food borne microorganisms. *Int. J. Food Microbiol.* 50: 119-130.

Cartuyvels R, Ridder C, Jonckheere S, Verbist L, van Eldere J. (1996). Prospective clinical evaluation of Amplicor *Mycobacterium tuberculosis* PCR test as a screening method in a low-prevalence population. *J Clin Microbiol* 34: 2001-2003.

Cooray, K. J., Nishibori, T., Xiong, H., Matsuyama, T., Fujita, M. and Mitsuyama, M. (1994). Detection of multiple virulence-associated genes of *Listeria monocytogenes* by PCR in artificially contaminated milk samples. *Appl. Environ. Microbiol*.. 60: 3023-3026.

Dominguez-Rodriguez, L., Suarez-Fernandez, G., Fernandez-Garayzobal, J. and Rodriguez-Ferri, E. (1984). New methodology for the isolation of *Listeria monocytogenes* from heavily contaminated environments. *Appl. Environ. Microbiol*. 47: 1188-1190.

Fluit, A. C., Torensma, R., Visser M. J. C., Aarsman, C.J.M., Poppeiler, M. J. J. G., Keller, B. H. I., Klapwijk, P. and Verhoef, J. (1993). Detection of *Listeria monocytogenes* in cheese with the magnetic immuno-polymerase chain reaction assay. *Appl. Environ. Microbiol*. 59: 1289-1293.

Furrer, B., Candrian, U., Hoefelein, Ch. and Luethy, J. (1991). Detection and identification of *Listeria monocytogenes* in cooked sausage products and in milk by *in vitro* amplification of haemolysin gene fragments. *J. Appl. Bacteriol.* 70: 372-379.

Gerstel, B., Gröbe, L., Pistor, S., Chakraborty, T. and Wehland, J. (1996). The ActA polypeptides of *Listeria ivanovii* and *Listeria monocytogenes* harbor related binding sites for host microfilament proteins. *Infect. Immun*. 64:1929-1936.

Gouws, P. A. and Liedemann, I. (2005). Detection of *L. monocytogenes* in Food Products. *Food Technol. Biotechnol. 43* (2): 201–205

Haas, A., Dumbsky, M. and Kreft, J. (1992). Listeriolysin genes: complete sequence of *ilo* from *Listeria ivanovii* and of *iso* from *Listeria seeligeri*. *Biochim. Biophys. Acta*, 1130: 81-84.

Herman, L.M.F., de Block, J.H.G.E. and Moermans, R.J.B. (1995) Direct detection of *Listeria monocytogenes* in 25 milliliters of raw milk by a two-step PCR with nested primers. *Appl. Environ. Microbiol*. 61: 817-819.

Hof, H. and Rocourt, J. (1992). Is any strain of *Listeria monocytogenes* detected in food a health risk? *Int.l J. Food Microbiol.*, 16: 173 182.

Kreft, J., Funke, D., Haas, A., Lottspeich, F. and Goepel, W. (1989). Production, purification and characterization of haemolysins from *L. ivanovii* and *L. monocytogenes.* Sv.4b.*FEMS Microbiol. Let.* **57**: 197-202.

Lantz, P., Tjerneld, F., Borch, E., Hahn-Hagerdal, B. and Radstrom, P. (1994) Enhanced sensitivity in PCR detection of *Listeria monocytogenes* in soft cheese through use of an aqueous two-phase system as a sample preparation method. *Appl. Environ. Microbiol*. 60, 3416-3418.

Makino, S., Okada, Y., & Maruyama, T. (1995). A New Method for Direct Detection of *Listeria monocytogenes* from Foods by PCR. *Applied and Environmental Microbiology*, 61, 3745-3747.

Mengaud, J., Vincente, M.F., Chenevert, J., Periera, J.M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.C. and Cossart, P. (1988). Expression in Escherichia coli and sequence analysis of listeriolysin O determinant of *Listeria monocytogenes. Infect. Immun.*, **56**: 766-772.

Niederhauser,C., Candrian, C., Hofelein, M., Buhler, H.P. and Luthy, J. (1992) Use of polymerase chain reaction for detection of *Listeria monocytogenes* in food. *Appl. Environ. Microbiol*. 58, 1564-1568.

Nishibori, T., Cooray, K., Xiong, H., Kawamuru, I., Fujita, M. and Mitsuyama, M. (1995) Correlation between the presence of virulence associated genes as determined by PCR and actual virulence to mice in various strains of *Listeria* spp. *Microbiol. Immunol*. 39, 343-349.

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

Olsen, J.E. (2000) DNA-based methods for detection of foodborne bacterial pathogens. *Food Res. Int*. 33, 257-266.

Paziak-Domanska, B., Bogulawska, E., Wiekowska-Szakiel, M., Kotlowski, R., Rozalska, B., Chmiela, M., Kur, J., Dabrowski, W. and Rudnicka, W. (1999) Evaluation of the API test, phosphatidylinositol-specific phospholipase C activity and PCR method in identification of *Listeria monocytogenes* in meat foods. *FEMS Microbiol. Lett*. 171, 209-214.

Portnoy, D.A., Chakraborty, T., Goebel, W. and Cassart, P. (1992) Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infect. Immun.* 60, 1263-1267.

Rawool, D.B., Malik, S.V.S., Shakuntala, I., Sahare, A.M. and Barbuddhe, S.B. (2007). Detection of multiple virulence-associated genes in *Listeria monocytogenes* isolated from bovine mastitis cases. *Int. J. of Food Microbiol*. 113:201-207.

Shakuntala, I., Malik, S.V.S., Barbuddhe, S.B. and Rawool, D.B., (2006). Isolation of *Listeria monocytogenes* from buffaloes with reproductive disorders and its confirmation by polymerase chain reaction. *Vet. Microbiol.* 117:229-234.

Suarez, M. and Vazquez-Boland, J. A. (2001) The bacterial actin nucleater protein a*ctA* is involved in epithelial cell invasion by *Listeria monocytogenes*. www.ncbi.nlm.nih.gov/pubmed [Accession No. AF103807].

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J. and Kreft, **J.** (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* 14: 584–640.

Wang, R.F., Cao, W.W. and Johnson, M.G. (1992) Development of cell surface protein associated gene probe specific for *Listeria monocytogenes* and detection of the bacteria in food by PCR. *Mol. Cell. Prob.* 6, 119-129

ADEWOYE , S.O. and OMOTOSHO ,J.S. (1997): Nutrient Composition of some freshwater fishes in *Nigeria Biosci. Res. Commun*. 11 (4) 333-336.

ADEWOYE, S. O., FAWOLE, O. O. and OMOTOSHO, J. S. (2003). Concentrations of selected elements in some fresh water fishes in Nigeria. *Science Focus*. Vol. 4, pp 106-108.

BENTLY, P. J. (1971). Endocrine and osmoregulation, Springer-Verdag Heidelberg. Pp. 220-230.

AKO, P.A. and SALIHU, S.O. (2004): "Studies on Some Major and Trace Metals in Smoked and Over-Dried Fish", *Journal of Applied Sciences and Environmental Management*, Vol. 8, No.2 Dec. pp 5-9.

A.O.A.C., (1975): Official Methods of Analysis 12th Edition. (W. Hortuntzed). Association of Official Analytical Chemists, Washington, D.C.

BOYD, C.E. and. DAVIS J.A (1978): Concentration of selected element and ash in Bluegill (Lepomis macrochirus) and certain other freshwater fish. *Trans. Am. Fish Soc.* 6: 862-867.

BURGRESS, G.H.O (1975): "Increasing the direct consumption of fish. In: WW Pirie (Edu). Food Protein Sources. *International Biological Programme* 4. Cambridge University Press, Cambridge, pp 187-200.

FORAN, J.A., CARPENTER, D. O., Hamilton, M.C., Knuth, B.A., and SCHWAGER, S.J., (2005): "*Risk-based consumption advice for farmed Atlantic and wild pacific salmon contaminated with dioxins and dioxin-like compounds*". *Environmental health perspective* 33:552-556.

KHAN, A.H., ALI, M., BIASWAS, S. K., and HADI, D.A. (1987): "*Trace elements in marine fish from the Bay of Bengal*". *The science of the total environment* 61: 12-130.

LADIPO, O. O., SONAIKE, O.O. and OLUDIMU, O.L. (1982): A statistical investigation of fish in Nigeria. Proceedings of the 2nd Annual Conference of fisheries society of Nigeria (FISON), Calabar, 25th -27th, January.

LAGLER, K.F., BARDACH, J.E. and. MILLER, R.R (1977): "Lethology, the study of fishes. Wiley, New York 156-163pg.

LENNTECH, (2006): "Lenntech Water Treatment and air purification holding B.V." Retrieved June 2006, from http://www.lenntech.com/feedback2.htm

MILLS, C.F. (1980): The mineral nutrition of livestock (Underwood, E.J. 1981 Ed.) Common Wealth Agricultural Bureaux Pg 9.

EIL, W., TREMBLAY and ANDREW, P. G. (1995): "*Human Health and Great lakes, and Environmental Pollution: A 1994 perspectives*". Retrieved June 26, 2006, from http://www.ehp.com.

SADIKU, S.O.E. and OLADIMEJI, A..A. (1991): "Relationship of proximate composition of Lates *niloticus* (L), *synodontis schall REs. Commun.* 3 (1), 29-40.

SHUL'MAN, G.E. (1974): Life cycle of fish: Physiology and Biochemistry, Halsted Press a division of John Wiley and Son Inc. N.Y. (1st Ed.) Pg 101-104.

TAYLOR, D.J.; GREEN, N.P.O. and. STOUT, G.W. (2002): *Biological Science*, 3rd edition, Cambridge CB21RP, Cambridge University Press.

WATERMAN, J..J. (2000): *Composition and Quality of Fish*, Edinburgh, Torry Research Station.

WINDOW H.; STEIN, D.; SCHELDON, R.; and SMITH, J. R. (1987): "Comparison of trace metal concentrations in muscle of a benthopelagic fish (Cory phaenoides armatus) from the Atlantic and Pacific oceans". *Deep seaResearch* 34 (2): 213-220.