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Comparative detection of Listeria monocytogenes in raw milk by microbiological method and PCR

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Summary

In this study, 150 raw milk samples from 15 different farms were collected in the city of Istanbul, and the raw milk samples were analyzed for Listeria monocytogenes by both conventional microbiological methods and PCR. According to the authors' results, 11 of 150 raw milk samples (7.33%) were detected to be Listeria monocytogenes positive by the both techniques. The results of the conventional microbiological analysis and the PCR procedures were parallel to each other. PCR must be used more frequently in food microbiology science, to protect public health and to prevent economic losses, because of its high rate of accurate results, time advantages, sensitivity and ability of producing desired DNA counts of the target microorganism.

Keywords: Listeria monocytogenes, PCR, milk

Listeria monocytogenes is an important facultative anaerobic pathogen that can cause sporadic and epidemic infections/outbreaks and can be isolated from almost all kinds of foods. Because the microorganism can exist in soil, mud, vegetables and vegetable products, milk and milk products and in waste waters, it can contaminate from animal to animal by fecal to oral route (5, 26). The major food vehicles for human listeriosis include milk and milk products, raw milk and raw milk products, cheese, meat and meat products and vegetables (7). Outbreaks of *Listeria monocytogenes* infections caused by the consumption of milk and milk products have been reported in many countries in the world, but mainly in the United States (4). *Listeria monocytogenes* is the main species that is responsible for human and animal listeriosis. Aside from *Listeria monocytogenes*, it is noted that *Listeria ivanovii* can also be a pathogen for humans (22). Although all the patients that have *Listeria monocytogenes* infection theoretically carry a death risk, pregnant, pediatric and geriatric cases, patients whose immune system are depressed and/or patients that have illnesses that can cause immune system depression, like HIV infection, hepatitis and cancer, are the primary risk group for listeriosis. *Listeria monocytogenes* can cause non-invasive or invasive listeriosis. Gastroenteritis with high fever is the main symptom of non-invasive listeiosis while invasive listeriosis causes meningitis, septicemia, primary bacteriemia, endocarditis, non-meningitic central nervous system infections, conjuctivitis and influenza like symptoms. Pattern births and abortions in pregnant cows and deaths by systemic infection in neonatals are also among the pathogenities of *Listeria monocytogenesis* (15). The listeriosis incidence in Austria for the year 2004 is 0.24% for every 100,000 citizens and the mortality rate of the patients that have listeriosis is 21% (2, 3).

Listeria monocytogenes, can frequently be isolated from unpasteurized milk and milk products and home made milk. While the agent is generally inactivated at pasteurization temperatures (8), it is indicated that *Listeria monocytogenes* can cross-contaminate to the equipment and/or to the milk and milk products by staff who carries the microorganism asymptomatically (27).

According to a study performed in the year 1987, milk samples from various sales points were collected and analyzed for *Listeria monocytogenes* in different countries. According to the results, 0.32% of the collected milk samples from Germany, 4.2% of the collected milk samples from France, 4.4% of the collected milk samples from Holland and 7% of collected milk samples from Massachusetts – USA were *Listeria monoctogenes* positive (12).

Conventional microbiological analysis methods are also being used to detect the existence of *Listeria monocytogenes* using *Listeria monocytogenes* specific media. However, conventional microbiological methods are

overly time consuming (1-3). Rapid analysis methods are important for microbiological quality detection of the foods. They are also quickly applied methods and they also have a high rate of accurate results. One of the most important and used rapid analysis microbiological methods is DNA based PCR, in spite of the fact that there are some analytical chemical methods such as vibrational spectroscopy and gas chromatography that have been successfully applied to the identification of bacteria for many years (10, 19, 25).

This study was performed to detect the existence of *Listeria monocytogenes* in the raw milk samples that are sold in İstanbul, by using both DNA based PCR and conventional microbiological analysis methods. Because the rapid analysis methods are becoming an alternative to the conventional microbiological methods, we also aimed to compare the results of these two different based methods.

Material and methods

Material. 150 raw milk samples collected from 15 different farms in Istanbul were used as material in this study. Samples were collected during the morning lactation and were put in sterile plastic boxes, after which they were transported to laboratories of İstanbul University, School of Veterinary Medicine, Department of Food Hygiene&Technology in aseptic conditions at 2-4°C. Each day 10 samples from a different farm were collected and all the samples were analyzed in the same conditions both by microbiological and PCR methods. All the raw milk samples were divided into two equal parts in the laboratory conditions. Each sample consisted of 200 ml. 100 ml of each sample was separated for microbiological analysis and the other 100 ml was separated for PCR analysis. Duplicate samples were directly analyzed by both microbiological methods and PCR.

Microbiological isolation and identification of *Listeria monocytogenes*. The Hitchins method (13) was used. Every sample was enriched with modified Listeria Enrichment Broth (Merck) (225 ml of Sterile Modified Listeria Enrichment Broth and 25 ml of raw milk sample). After the enrichment procedure, all the enriched samples were homogenized 3 minutes in Stomacher (Seward Laboratory, London, United Kingdom). Homogenized samples were incubated 48 hours at 30°C. After the incubation period was completed, all the enriched samples were inoculated onto Modified Oxford Agar (Merck) and Palcam Agar (Merck) plates by round ended loop, and the samples were again incubated 48 hours at 35°C. Greenish colonies with black zones or brownish-green colonies with a dark gray zone were evaluated as suspected colonies for identification. 5 typical colonies from every media were passed to Triptic Soy Agar (Merck) plates with 0.6% Yeast Extract (Merck) and the samples were incubated 48 hours at 30°. Positive samples were analyzed for morphological inspection, motility, gram staining, catalase production and hemolitic activity in Blood Agar with 5% sheep blood. For confirmation, nitrate reduction, production of urease, H_2S production in Triple Sugar Iron Agar (TSIA), MR – VP reaction, esculin hydrolisis, acid production activity in dextrose, maltose, rhamnose and xylose (DIFCO, Detroit, Michigan, USA) analyses were performed on all the positive suspected samples. The isolates given typical *Listeria monocytogenes* reactions were analyzed with polyvalant „O" antiserum (DIFCO) for slide aglutination test and serotyping procedure was performed. Suspected colonies were aglutinated with somatic (type 1 and 4) and flagellar (AB) monovalant serums according to BAM – FDA method (13).

PCR Procedure

Bacterial strains. The bacterial strains used in this study are shown in table 1. Liophylized strains were incubated in Triptic Soy Broth (Merck) with 0.6% Yeast Extract (Merck) 48 hours at 30°C.

Tab. 1. The bacterial strains used in the study

Name of the bacteria	Strains	Serotype*	Isolation**
L. monocytogenes	ATCC19113	3	Human
L. monocytogenes	ATCC19114	4a	–
L. monocytogenes	ATCC19115	4b	Human
L. monocytogenes	ATCC19117	4d	Sheep
L. monocytogenes	ATCC19118	4e	Chicken
L. monocytogenes	ATCC35152	–	Guinea Pig

Explanations: * – source: http://www.atcc.org; ** – source: http://www.atcc.org

Artificially contaminated milk samples. Milk samples were obtained from local supermarkets as UHT pasteurized milk samples. Additionally, raw milk samples that were contaminated artificially were provided from the Research Farm of the School of Veterinary Medicine, İstanbul University. To find out the most suitable DNA isolation method, all the provided samples that were to be contaminated artificially (both raw and pasteurized milk samples), were enriched with Listeria Enrichment Broth (Merck) at a ratio of 25 ml milk sample and 225 ml Listeria Enrichment Broth. Next, the samples were contaminated with 2.5 ml of pure *Listeria monocytogenes* colonies in Ringer solution (Merck) with a dilution of 10^1 concentration and contaminated samples were incubated 48 hours at 30°C. The dilution series were set in order to create 1-10, 10-100 and 100-1000 cfu/ml concentrations. After the inoculation period, enriched samples were passed onto Modified Oxford Agar (Merck) and Palcam Agar plates (Merck), the samples were again incubated for 48 hours at 35°C. After the incubation process, the colonies were inoculated onto Triptic Soy Agar plates (Merck) with 0.6% Yeast Extract (Merck) and the samples were incubated at 48 hours at 30°C.

DNA extraction. The following method was applied to all the raw milk samples. Modified CTAB/Proteinase K method was used in the DNA extraction procedure (29). Suspected colonies were inoculated onto the media containing 200 µl TE buffer, 60 µl 10% Sodium dodecyl sulphate (SDS) and 5 µl 20 mg/ml proteinase K and incubated overnight at 65°C. 100 µl 5 M NaCl and 80 µl CTAB/NaCL solutions were added to eppendorf tubes and the eppendorf tubes were incubated 10 minutes at 65°C. 500 µl Phenol/chloroform/Isoamyl alcohol (25 : 24 : 1) were added to the eppendorf tubes. The tubes were mixed and cenrifuged

Tab. 2. The primers used in the study

Primers	Sequences (5'-3')	Polarity	T_m Values (°C)
lap F: (LIM 2)	CTA AAG CGG GAA TCT CCC TT	Sense	60
lapR: (LIMRE)	CCA TTG TCT TGC GCG TTA AT	Antisense	62
DG 67	CCTGCAAGAGCTCAGACGCC	Sense	66
DG 68	CGTAAGGATCCGAGGTTGCC	Antisense	64
DG 69	GTGCCGCCAAGAAAAGGTTA	Sense	60
DG 70	CCTTCACGATTGCGCCGAA	Antisense	62
DG 71	CGGAGGTTCCGCAAAAGATG	Sense	62
DG 72	CCTCCAGAGTGATCGATGTT	Antisense	60
hlyAF: (DG73)	GAC ATT CAA GTT GTG AA	Sense	46
hlyAR: (DG74)	CGC CAC ACT TGA GAT AT	Antisense	50
DG 75	GACCGCAAGGTTGAAACTCA	Sense	60
DG 76	CAGCCTAACAATCCGAACTGA	Antisense	60

Explanations: T_m values, T_m (°C) = 2 (A+T) + 4 (G+C) (Sambrook and Russel, 2001); source: http://www.atcc.org

5 minutes. After centrifugation, supernatant was removed. Moreover, for removing phenol traces, 500 µl chloroform/ Isoamyl alcohol (24 : 1) was added to the media. DNA samples were precipitated 0.6 ml pure ethanol solution at – 20°C for precipitation. The mixture was then centrifuged at maximum rotations per minute for 10 minutes. After centrifugation pure ethanol solution removed and DNA was provided with the application of 30 µl TE buffer.

Primers. Various primer pairs were used to detect specific gene fragments of *Listeria monocytogenes* (31). To detect the most suitable primer/primers, all the primers were evaluated individually. The amplicon size was 560 bp (Thermo Hybaid Hbpx 220 CE 27812, England) (Elizabeth, Thomas King, Burchak and Gannon 1991). The primers used in the study are indicated below in table 2.

PCR procedure. PCR reaction mixture consisted of 10 mM Tris-HCL (pH 8.3), 50 mM KCl, 1.5 mM KCl_2, 200 µM dNTP, 2.5 unit of Taq DNA polymerase and was prepared 100 pmol for every primer. Samples were denaturated at 94°C, 5 minutes at thermocycler amplification. Every cycle consisted of 45 seconds of denaturation levels at 94°C. 45 seconds of an annealing step at 55°C and 45 seconds of an extension step at 72°C were applied to the samples. Finally, the last extension step (5 minutes at 72°C, 30 ampflication cycles) was applied. PCR results were evaluated in agar gel electrophoresis of 1.5% agarose gel with ethidium bromide.

Results and discussion

Microbiological results. According to the results, 7.33% of the analyzed samples were *Listeria monocytogenes* positive (11 samples *Listeria monocytogenes* positive of 150 samples). The results of both the microbiological analysis and PCR analysis were parallel to each other for each analyzed raw milk sample. The obtained strains were inoculated according to the method of Hitchins (13). All the raw milk suspected colonies detected from the raw milk samples were confirmed (especially at the chemical test stages) with the strains of the original ATCC species, both by conventional microbiological methods and PCR procedures. *L. monocytogenes* colonies with green/blue reflection at 45° light transmission incidence of Henry (12). The positive result samples were evaluated as *Listeria monocytogenes* and again the positive samples were analyzed with PCR procedure for a positiveness comparison.

PCR results. According to the results, which were parallel to the microbiological results, 7.33% of the analyzed samples were *Listeria monocytogenes* positive (11 samples were *Listeria monocytogenes* positive from the 150 samples). The negative and positive results of both microbiological analysis and PCR analysis were parallel to each other with each raw milk sample. 11 identical samples of the collected raw milk were positive according to the conventional microbiological methods and PCR procedures. 139 identical samples of collected raw milk gave negative results according to both methods. The primers used in PCR procedure are indicated in table 2. Six primer sets were used to determine the most suitable/effective primer set/sets. Band densities of the various primer sets were quite different. DG 73 and DG 74 primer sets were evaluated as the most effective primer sets for our study. Also DG 73 and DG 74 produced more DNA copies than the other primer sets (fig. 1); moreover, since the mentioned primers were the most effective ones, DG 73 and DG 74 primer sets were used for all the samples for all of the reported PCR procedures and results.

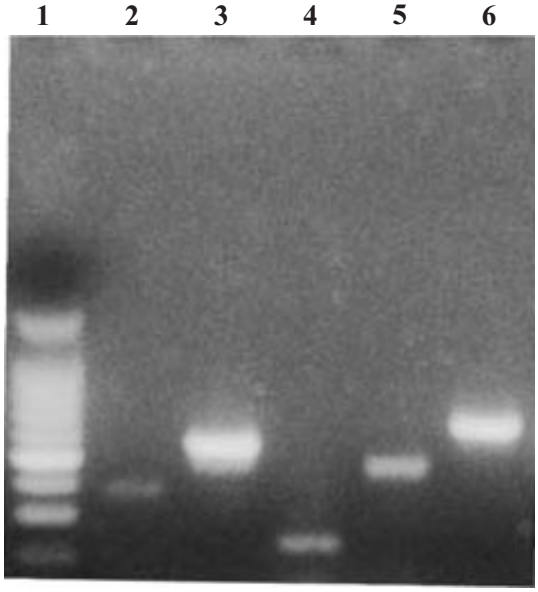

Fig. 1. DNA profiles of 5 different primer sets at gel electrophoresis
Explanations: Lane 1: Standart DNA (100 bp); Lane 2: DG70/DG73 (386 bp); Lane 3: DG73/DG74 (560 bp); Lane 4: DG71/DG72 (234 bp); Lane 5: DG69/DG70 (470 bp); Lane 6: DG69/DG74 (636 bp)

To measure PCR sensibility, 1 ml of the UHT milk samples obtained from local supermarkets and raw milk samples obtained from the Research Farm of the School of Veterinary Medicine, İstanbul University, were artificially contaminated with *Listeria monocytogenes* with a concentration of 10^1 to 10^7 cfu/ml respectively. During the artificial contamination procedure, DNA extraction and amplification was directly applied to the samples. The limit values were detected as 10^3 cfu/0.5 ml (fig. 2).

The increase of the PCR cycle did not effect the PCR sensitivity. However, TSYB adding procedure to the samples diluted up to 20 times, and incubation procedure after adding TSYB at 25°C 24 hours, affected the PCR sensitivity positively and even one microorganism could be detected (fig. 3).

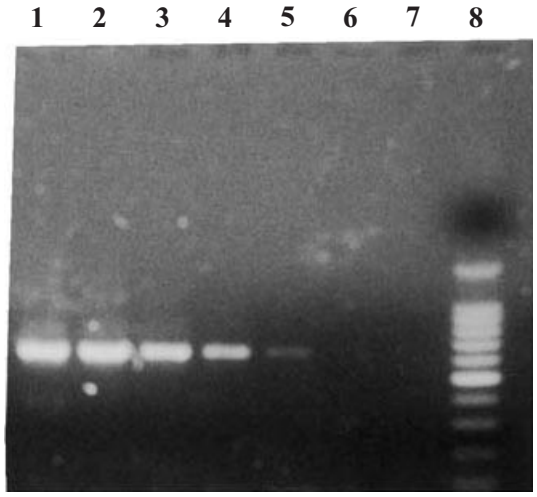

Fig. 2. The detection of *Listeria monocytogenes* limit in milk. In PCR procedure, the microorganisms up to 10^3 cfu/ml concentrations could be observed clearly, Lower concentrations than 10^3 cfu/ml could not be observed clearly (lane 6 and lane 7). Lane 8 shows standart DNA chain, Lane 1 is the concentration of 10^7 cfu/ml

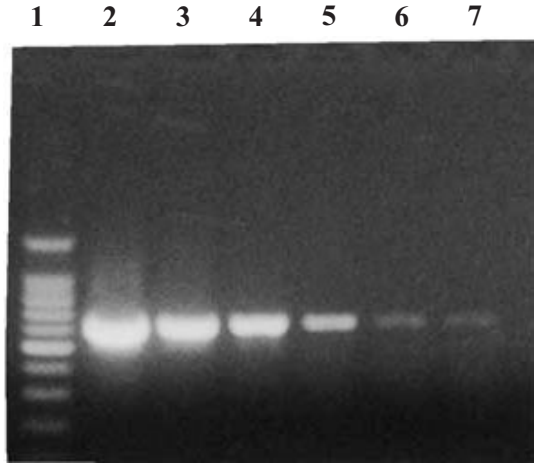

Fig. 3. The limit and sensitibility test to the samples TSYB added. Up to 20 times TSYB diluted samples incubated at 25°C 24 hours and DNA extraction and PCR procedure was applied to the samples after incubation period. Lane 1 shows the standart DNA chain, lane 2 shows the concentration of 10^5 cfu/ml, lane 3 shows the cocncentration of 10^4 cfu/ml, lane 4 shows the concentration of 10^3 cfu/ml, lene 5 shows the concentration of 10^2 cfu/ml, lane 6 shows the cocncentration of 10^1 cfu/ml and finally lane 7 shows the cocncentration of 1 cfu/ml

In this study has the goal of detecting the existence of *Listeria monocytogenes* in raw milk samples collected from different milk farms using conventional microbiological methods as well as PCR and to compare these two scientific methods to each other. PCR is also applied by a good number of projects supported by the European Union as an alternative of the conventional microbiological methods because of its high and trustworthy rate of results (19).

İstanbul is the most crowded and the biggest city of Turkey. The city has a population of approximately 15 million and milk and its products are widely consumed. Medical literature indicate that the existence of *Listeria monocytogenes* in raw milk samples increases especially in March, April and June (30). For this reason we, too, collected the raw milk samples from 15 different milk farms at the end of March and at the beginning of April. *Listeria monocytogenes* also has a high cross-contamination rate from unhygienic equipment to animals and/or human (24). Additionally, it is indicated that *Listeria monocytogenes* can resist even UHT pasteurization processes by infiltrating the somatic cells and/or leukocytes in the raw milk (14, 21). Unhygienic stables, careless farm staff, collection of the raw milk obtained from various farms in common milk tanks are factors that increase the risk of contaminaton. One of our experiments was to detect the time versus cfu forming speed of the agent in the laboratory conditions. We inoculated 11 *Listeria monocytogenes* colonies that we have obtained from 11 positive samples to 11 different Modified Oxford Agar (Merck) and Palcam Agar (Merck) media at the concentration of 1 cfu/ml for each positive raw milk sample after completing both PCR and conventional microbiological analysis for detecting the growth speed of *Listeria monocytogenes*; after 24-36 hours we observed that the number of cfu reached the concentration of 10^5-10^6 counts. In spite of the fact that we currently have no knowledge of the minimal infection dose of *Listeria monocytogenes*, according to the medical literature we can clearly point out that the concentration of *Listeria monocytogenes* can easily reach 10^5-10^6 cfu/ml in as short a time interval as 24-36 hours. According to this information we detected, we think that *Listeria monocytogenes* infected raw milk carries a serious risk for consumer health.

Listeria monocytogenes is also known to be a toxigenic microorganism. Listeriolysin O (LLO) has been shown to be a virulence factor in *L. monocytogenes* and is not found in other *Listeria spp.* The in vitro studies significantly improved the efficiency of liposome-mediated cytosolic delivery by co-encapsulating purified listeriolysin O (LLO) inside liposomes and pH-sensitive liposomes. LLO is the major virulence

factor and causative component for phagosomal escape of the facultative intracellular bacteria *Listeria* into the cytosol of host cells (23). Even at UHT temeperatures the LLO can not be denaturated (18). According to the authors, this situation is a critical control point for consumer health because of the pathogenitiy of LLO. One of PCR's advantages according to the conventional microbiological method is to detect the microorganism even if it is inactivated. If the microorganism is inactivated with heating and/or other procedures as UV, centrifuging, etc., it is impossible to determine the existence of *Listeria monocytogenes* and other pathogens by microbiological methods; however, like other toxigenic pathogens *Listeria monocytogenes* can produce LLO before it is inactivated and the toxins can exist in milk and its products even if the microorganism does not exist in the milk and its products. On the other hand we would like to indicate that home made milk and home cheese is widely produced, especially in rural regions in Turkey. So we think that PCR is a good alternative especially for determining the existence the toxigenic pathogens in foods to the conventional microbiological methods.

The low percentage of existence of *Listeria monocytogenes* (generally lower than 10%) is also widely indicated by the medical literature, too. Parallel to the medical literature, the percentage rate of *Listeria monocytogenes* that we detected in our study was 7.33% (11 *Listeria monocytogenes* positive raw milk samples/150 total raw milk samples). However, according to the authors it must not be forgotten that the some milk/milk products sourced *Listeria monocytogenes* outbreaks that resulted in fatal cases were caused by milk products that are pasteurized, like soft Mexican cheese (9, 16). Loncaveric et. al. (17) and Margolles et al. (20), point out that *Listeria monocytogenes* is especially a major risk factor in milk products (generally in cheese) that were applied to short term aging processes and/or pasteurized under weak hygienic conditions. According to the available information, the authors think that a final product microbiological analysis for *Listeria monocytogenes* must be conducted particularly to milk and its products. *Listeria monocytogenes* can additionally resist refrigerator temperatures and easily grow, too (24). According to the authors, these acceptable values carry serious risks for consumer health. Moreover, *Listeria monocytogenes* can cross-contaminate with unsuitable disinfectant chemicals (28). The authors are of the opinion that PCR must be used more frequently in food microbiology science, to protect public health and to prevent economic losses, because of its high rate of accurate results, time advantages, sensitivity and ability of producing desired DNA counts of the target microorganism.

References

1. *Anon*.: ISO 11290-1: Microbiology of Food and Animal Feding Stuffs Horizontal Method for the Detection and Enumeration of Listeria monocytogenes, Part 1: Detection Method, Geneva 1996.
2. *Anon*.: [in:] ISO 11290-1/Amd. 1: Modification of the Isolation Media and the Hemolysis Test, and Inclusion of Precision Data, Geneva, Switzerland.
3. *Anon*.: Nationale Referenzzentrale für Listerien Jahresbericht Mitt. Sanitätsverw. 106, 19-24.
4. *Beckers H. J., Soentoro P. S. S., Delfgou-van Asch. E. H. M*.: The occurrence of Listeria monocytogenes in soft cheeses and raw milk and its resistance to heat. Int. J. Food Microbiol. 1987, 4, 249-256.
5. *Brackett R. E*.: Presence and persistence of Listeria monocytogenes in food and water. Food Technol. 1988, 42, 162-164.
6. *Elizebeth J., Thomas G., King R. K., Burchak J., Gannon V. P. J*.: Sensitive and specific detection of Listeria monocytogenes in milk and Ground beef with the Ploymerase Chain Reaction. Appl. Environ. Microbiol. 1991, 57, 2576-2580.
7. *Farber J. M., Peterkin P. I*.: Listeria monocytogenes, a food-borne pathogen. Microbiol. Rev. 1991, 55, 476-511.
8. *Fernandez-Garayzabal J. F., Dominguez L., Vazquez J. A., Blanco J. L., Suarez G*.: Listeria monocytogenes dans le lait pasteurise. Can. J. Microbiol. 1986, 32, 149-150.
9. *Fleming D. W., Cochi S. L., MacDonald K. L., Brondum J., Hayes P. S., Plikaytis B. D., Holmes M. B., Audurier A., Broome C. V., Reingold A. L*.: Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. 1986, 312, 404-407.
10. *Fox A*.: Carbohydrate profiling of bacteria by gas chromatography-mass spectrometry and their trace detection in complex matrices by gas chromatography-tandem mass spectrometry. J. Chromagr. 1999, 843, 287-300.
11. *Gaya P., Sánchez J., Medina M., Núñez M*.: Incidence of Listeria monocytogenes and other Listeria species in raw milk produced in Spain. Food Microbiol. 1998, 15, 551-555.
12. *Hird D. W*.: Review of evidence for zoonotic listeriosis. J. Food. Prot. 1987, 50, 429-433.
13. *Hitchins A. D*.: FDA Bacteriological Analytical Manual. AOAC International, Gaithersburg, MD, USA 2003, 10.01-10.13
14. *Hitchins A. D*.: Assessment of alimentary exposure to Listeria monocytogenes. Int. J. Food Microbiol. 1996, 30, 71-85.
15. ILSI Expert Panel. Achieving continuous improvement in reductions infoodborne listeriosis a risk-based approach. J. Food Prot. 2005, 68, 1932-1994.
16. *Linnan M. J., Mascola L., Lou X. D., Goulet V., May S., Salminen C., Hird D. W., Yonekura M. L., Hayes P., Weaver R., Audurier A., Plikaytis B. D., Flannin S. L., Kleks A., Broome C. V*.: Epidemic listeriosis associated with Mexican-style cheese. N. Engl. J. Med. 1988, 319, 823-828.
17. *Loncaveric S., Danielsson-Tham M. L., Tham W*.: Occurrence of Listeria monocytogenes in soft and semi-soft cheeses in retail outlets in Sweden. Int. J. Food Microbiol 1995, 26, 245-250.
18. *Low J. C., Davies R. C., Donachie W*.: Purification of listeriolysin-O and development of an immunosassay for diagnosis of listeric infections in sheep. J. Clin. Microbiol. 1992, 30, 2705-2708.
19. *Malorny B., Tassios P. T., Radström P., Cook N., Wagner M., Hoorfar J*.: Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol. 2003, 83, 39-48.
20. *Margolles A., Rodríguez A., de-los-Reyes-Gavilan C. G*.: Some chemical and bacteriological characteristics of regional cheeses from Asturias, Spain. J. Food Prot. 1996, 59, 509-515.
21. *McLauchlin J. R. T*.: The relationship between Listeria and listeriosis. Food Control 1996, 7, 187-193.
22. *McLauchlin J., Mitchell R. T., Smerdon W. J., Jewell K*.: Listeria monocytogenes and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. Int. J. Food Microbiol. 2004, 92 (1), 15-33.
23. *Miller L., Kaper J. B., Portnoy J. B., Isberg R. R*.: Molecular Genetics of bacterial pathogenesis. ASM Press, Herndon, USA 1994.
24. *Norrung B*.: Microbiological criteria for L. monocytogenes in foods under special consideration of risk assessment approaches. Int. J. Food Microbiol. 2000, 62, 217-221.
25. *Norton D. M*.: Polymerase chain reaction-based methods for detection of Listeria monocytogenes: Toward real-time screening for food and environmental samples. J. AOAC Int. 2002, 85, 505-515.
26. *Pinner R. W., Schuchat A., Swaminathan B., Hayes P. S., Deaver K. A., Weaver R. E., Plikaytis B. D., Reeves M., Broome C. V., Wenger J. D*. and the Listeria study group: Role of foods in sporadic listeriosis: II. Microbiological and epidemiologic investigation. J. Am. Med. Assoc. 1992, 267, 2046-2050.
27. *Potter M. E., Kaufmann A. F., Feldman P. A*.: Unpasteurized milk: the hazards of a health fetish. J. Am. Med. Assoc. 1984, 252, 2048-2052.
28. *Salvat G., Toquin M. T., Michel Y., Colin P*.: Control of Listeria monocytogenes in delicatessen industries: the lessons of a listeriosis outbreak in France. Int. J. Food Microbiol. 1995, 25, 75-81.
29. *Sambrook J., Russel D. W*.: Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, USA 2001.
30. *Yoshida T., Kato Y., Sato M., Hirai K*.: Sources and routes of contamination of raw milk with Listeria monocytogenes and its control. J. Vet. Med. Sci. 1998, 60, 1165-1168.
31. *Weon S. C., Hong C. H*.: Rapid enumaration of Listeria monocytogenes in milk using competetive PCR. Int. J. Food Microbiol. 2003, 84, 79-85.

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