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ORIGINAL

RAPID DNA EXTRACTION AND PCR VALIDATION FOR DIRECT DETECTION OF *Listeria monocytogenes* IN RAW MILK

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ABSTRACT

Objective. The aim of this study was to validate a method for detecting *L. monocytogenes* in raw milk. **Materials and methods**. The extraction procedure carried out using a chaotropic agent like NaI, to reduce fat in the sample to 0.2% w/v, which is the lowest limit for detection in the Gerber method, to avoid the polymerization. The raw milk samples were analyzed by using the traditional gold standard method for *L. monocytogenes.* Detection PCR was done on the specificity of primers that recognize the *Listeria* genus by amplifying a specific fragment of about 938bp of the 16S rDNA. Several primer sets were use: L1 (CTCCATAAAGGTGACCCT), U1 (CAGCMGCCGCGGTAATWC), LF (CAAACGTTAACAACGCAGTA) and LR (TCCAGAGTGATCGATGTTAA) that recognize the *hly*A gene of *L. monocytogenes*, amplifying a 750bp fragment. **Results.** The DNA of 39 strains evidenced high specificity of the technique since all the strains of *L. monocytogenes* amplified the fragments 938bp and 750bp, specifically for genus and species, respectively. The detection limit of the PCR was 10^1 CFU/ml. T he PCR reproducibility showed a Kappa of 0.85; the specificity and sensitivity of 100% were found, predictive positive and negative values were of 100% respectively. **Conclusions**. These results demonstrate that is possible to detect of *Listeria* spp. by using any of the three methods since they share the same sensitivity and specificity. One hundred percent of the predictive value for PCR (alternative method) provides high reliability, and allows the detection of the positive samples. The extraction procedure combined with a PCR method can reduce in 15 days the time of identification of *L. monocytogenes* in raw milk. This PCR technique could be adapted and validated to be use for other types of food such as poultry, meat products and cheeses.

Key words: *Listeria monocytogenes,* PCR, validation, raw milk.

EXTRACCIÓN RÁPIDA DE DNA Y VALIDACIÓN POR PCR PARA LA DETECCIÓN DIRECTA DE *Listeria monocytogenes* EN LECHE CRUDA

RESUMEN

Objetivo. Validar un método para la detección directa de *L. monocytogenes* en leche cruda. **Materiales y métodos**. Se utilizó un procedimiento de extracción con el agente caotropico NaI, para reducir la grasa en la muestra a un 0.2% p/v, el cual es mas bajo limite de detección con el método de Gerber para evitar la polimerización. Las muestras de leche cruda fueron analizadas por el método estandar de oro para la

detección de *L. monocytogenes.* La detección por PCR fue realizada amplificando el segmento específico de 938bp de 16S ADNr. Los siguientes cebadores fueron utilizados L1 (CTCCATAAAGGTGACCCT), U1 (CAGCMGCCGCGGTAATWC), LF (CAAACGTTAACAACGCAGTA) and LR (TCCAGAGTGATCGATGTTAA) que reconoce el gen *hly*A de *L. monocytogenes* y que amplifica un fragmento de 750bp.**Resultados.** El ADN de 39 cepas evidenció una alta especificidad de la técnica ya que todas las 39 *L. monocytogenes* amplificaron los fragmentos de 938bp y 750bp especie especifica. El límite de detección de la PCR fue de 10^1 CFU/ml. La reproducibilidad de la PCR mostró un Kappa de 0.85, la especificidad y sensibilidad fueron del 100%. El valor predictivo positivo y negativo fue del 100%. **Conclusiones**. Los resultados demostraron que es posible detectar *Listeria* spp usando cualquiera de los tres métodos ya que poseen la misma sensibilidad y especificidad. El 100% del valor predictivo positivo provee una alta posibilidad de detección en muestras positivas con listeria. EL método de extracción y la PCR estandarizada permiten en 15 días detectar *L. monocytogenes* en leches crudas. La técnica de PCR puede ser adaptada y validada otros tipos de alimentos como pollos, carnes y quesos.

Palabras clave: *Listeria monocytogenes,* PCR, validación, leche cruda.

INTRODUCTION

Listeria monocytogenes has been recognized as an important transmission route leading to human listeriosis, becoming a public health problem. It is a food-borne pathogen, responsible for severe and fatal infections. This microorganism can enter into the food chain through animals that shed cells in their milk and faeces. The organism, a Gram-positive, psychotropic rod, is widespread in nature and can be isolated from a variety of sources, such as poor quality silage, vegetation, soil, sewage, stream water, mud, slaughterhouse waste, milk of normal cows, and faeces of healthy humans. Additionally, cells of *Listeria* spp. have been isolated from 37 species of mammals, and 17 species of birds, as well as from flies, ticks, fish and crustaceans (1). The incidence of *L. monocytogenes* in humans is relatively low when compared with other diseases, while the mortality rate is approximately 30%. Among the illnesses associated with listeriosis are: meningitis, encephalitis, septicemias and abortions (2,3). The most susceptible population groups are those that have reduced cellular immunity, such as newborns, pregnant women, elderly people, Cancer and AIDS patients, and alcoholics (4, 5).

The incidence of *L. monocytogenes* in milk was studied in Colombia in the year 1996. Results showed an incidence of 34% in raw milk and of 2% in pasteurized milk; these reports are high when compared to those carried out in industrialized countries such as The Netherlands, France (4.6%) and the United States (12%) (6). Recent studies carried out by the Microbial and Food Ecology Laboratory of The Universidad de Los Andes have reported the prevalence of *L. monocytogenes* in different kinds of cheese of approximately 29.6% (7). Worldwide reports on listeriosis have increased in the last few years due to the growing number of people who may acquire this illness, and to the increased consumption of dairy products that frequently transmit the disease (8,9).

The routine method for detecting *L. monocytogenes* in food samples involves the use of selective enrichments and subsequent culturing on selective media; it is a laborious and time-consuming procedure. Therefore, a rapid, sensible and reliable method for *L. monocytogenes* detection is desirable (10,11). The aim of this study was to validate a method for removing PCR inhibitory factors, like milk fat, and other PCR techniques for differentiating *L. monocytogenes* from other *Listeria spp*. In order to find out the usefulness of combining an extraction procedure with the PCR method. The aim of this study was to validate a method for detecting *L. monocytogenes* in raw milk.

MATERIAL AND METHODS

Strains and master mell banks (MCB)

Sixteen bacterial genera distributed in 39 strains were used: 6 strains of *Listeria* and 33 of other contaminant and/or normal biota of raw milk (Table 1). All the strains were preserved in culture media, supplemented with glycerol at 30% (v/v) and stored at -70°C. The whole MCB was followed up with viability and microbial purity tests every two months of conservation (12,13).

Table 1. Microorganisms used in the study.

No.	Strains	Source
1	*L. monocytogenes*	Isolated from raw milk
2	*L. innocua*	
3	*Oenococcus oeni*	Food Microbiology Lab. Collection
4	*Salmonella typhi*	
5	*Bacillus cereus*	ATCC 10876
6	*Clostridium perfringens*	ATCC 12915
7	*Salmonella enteriditis*	ATCC 13076
8	*L. monocytogenes*	ATCC 1915
9	*L. monocytogenes*	ATCC 4640
10	*Escherichia coli*	ATCC 8739
11	*Pseudomonas aeruginosa*	ATCC 9027
12	*L. monocytogenes*	ATCC4334
13	*Escherichia coli* O126	
14	*E. coli* O127	
15	*Salmonella bredeney monophila*	
16	*S. colera suis var kuzendorf*	
17	*S. enteritidis*	
18	*S. infantis*	
19	*S.paratyphi A*	CDC Atlanta
20	*S. paratyphi B*	
21	*S.paratyphi C*	
22	*Shigella A8a8c*	
23	*Shigella B2b*	
24	*Shigella B4a*	
25	*Vibrio cholerae 1518-65*	
26	*Bacillus lincheniformis*	Applied Biotechnology Lab collection
27	*Escherichia coli*	
28	*Lactococcus lactis*	Food Microbiology Lab collection
29	*Micrococcus luteus*	
30	*Listeria innocua suiza*	
31	*Proteus* sp.	
32	*Bacillus subtilis*	
33	*Enterococcus faecalis*	
34	*Klebsiella sp.*	Microbiology Lab collection
35	*Salmonella* spp.	
36	*Staphylococcus aureus*	
37	*S. epidermidis*	
38	*Lactobacillus bavaricus*	Microbiology Lab collection
39	*Lactobacillus plantarum*	(UNIVALLE)

Extraction and quantification of genomic DNA

Gram-negative strains were cultivated in Luria Bertani broth (tryptone 10g/l, yeast extract 5g/l, NaCl 10g/l) with shaking. Gram-positive strains were cultivated in Columbia broth (special nutritional substrate 23g/l, starch 1g/l, NaCl 5g/l, agar-agar 15g/l) for 12 hours at 37°C and at 250 rpm. One milliliter of culture was centrifuged for 10 minutes, according to Gram identification: 2000g for Gram-positive and 500x g for Gram-negative microorganisms. Cells were washed in TE 1X buffer (Tris-HCl 10mM, EDTA 1mM pH 8.0 ± 0.2). Pellet was incubated in 200µl of TE 1X buffer with 2mg/ml of lysozyme for 30 min at 37°C for cell lysis. 300µl of TE 1X buffer with 1% (w/v) of SDS and 100µg/ml of proteinase K were added; the mixture was incubated at 65 °C during 1 hour for protein degradation. Residual peptides and lipids were removed by adding 84µl of NaCl 5M and 60µl CTAB (CTAB 10% (w/v) dissolved in 0.7M of NaCl), for 20 min at 65 °C. The resultant suspension was treated with a 24:1 mixture of chloroform/isoamilic. The aqueous phase was then treated with 2-propanol to precipitate cell DNA. The DNA was washed in 70% (v/v) of ethanol and suspended in TE 1X buffer. DNA purity and concentration were assayed in a Biospec 1601 Shimadzu spectrophotometer (260/280nm) with background correction at 320nm (14,15).

DNA electrophoresis

The extracted DNA and PCR reaction products were separated electrophoretically in 1% (w/v) agarose gel, prepared in 1X TAE buffer (40mM Tris-acetate, 1mM EDTA pH 8.0 ± 0.2). Gels were stained with etidium bromide 5µg/ml; 120 volts for 1h were the run conditions. Gels photograph were took under ultraviolet light. As molecular size marker, 100pb Ladder (Invitrogene™) was used (14,16).

PCR reaction

Two primer sets were used: L1 (CTCCATAAAGGTGACCCT) and U1 (CAGCMGCCGCGGTAATWC), that recognize the *Listeria* genus amplifying a fragment of about 938bp of the 16S rDNA; and LF (CAAACGTTAACAACGCAGTA) and LR (TCCAGAGTGATCGATGTTAA) that recognize the *hly*A gene of *L. monocytogenes*, amplifying a 750bp fragment. The final volume of the PCR reaction was 50µl, and the mixture was composed of 1X PCR buffer, 1.5mM of $MgCl_2$, 0.2mM (each) dNTP, 20pmol of primer, and 2U TaqDNA polymerase (Invitrogene™). Five µl of DNA (~200ng) of the extracted sample were used. Temperature cycling was controlled in a BioRad Gene Cycler™ programmed as follows: 95°C for 1min, followed by 30 cycles of 94°C for 30s, 51°C for 20s, 72°C for 30s and a final extension step at 72°C for 8min (15,17,18).

Steps for PCR validation

Physical and chemical analysis of the raw milk samples

Twenty samples of raw milk were submitted to physical and chemical analyses according to the techniques recommended by the National Health Institute of Bogotá Colombia: alcohol test, pH, percentage of lactic acid, reductase assay, and tests for starch, sucrose, chlorides, formaldehyde and fatty detection, etc.

Microbiological analysis of the raw milk samples

The raw milk samples were analyzed by using the traditional Gold Standard method for *L. monocytogenes* detection (19). Briefly, 25ml of raw milk were taking to a final volume of 250ml with enrichment broth for *Listeria* (Palcam broth); the suspensions were incubated at 30°C for 48 hours. Later, the cultures were plated on Palcam agar and incubated at 30°C from 24 to 48h. After the incubation time, the positive colonies for esculine hydrolysis were selected and then re-isolated on TSAYE agar (tryptone soy agar, yeast extract) and incubated at 30°C for 24h. Colonies were Gram and catalyze tested. A second purification was made in TSAYE agar, and incubated at 35°C for 24h; later on, Henry's illumination test was made; positive colonies for this test were assayed for motility, CAMP, RMVP and fermentation of mannitol, ramnose and xylose (10,20).

Reproducibility determination of the PCR method

The reproducibility test started with a pilot analysis with 15 replications of the PCR detection for *L. monocytogenes* ATCC 1915, *L. innocua (Swiss), Staphylococcus aureus* ATCC 29213 and *Escherichia coli* O127, followed by 3 replications of the PCR for each one of the 39 strains of the study.

Preliminary determination of the PCR densitivity and comparison with the gold standard and the immunological methods

PCR, Gold Standard Method and an immunological method (available in Colombia) were compared through double-blind assay. For this purpose, 15 screw cap tubes with 5ml of tryptone soy broth were artificially contaminated with 10^8CFU/ml or equivalent to tube 5 of the MacFarland scale; the strains employed for this assay were *L. monocytogenes* ATCC 1915, *L. innocua* (Swiss), *E. coli* O127 and *S. aureus* ATCC 29213. The samples were processed following the methodology described for each one of the methods mentioned above; later on, a statistical analysis was made.

DNA extraction from raw milk samples

200µl of raw milk, homogenate by vortex sample, were mixed with 400µl of lysis buffer (0.5% (w/v) *N*-laurylsarcosine, 50mM Tris-HCl, 25mM EDTA, pH 8.0 ± 0.2). After vortexing for 1min, the mixture was centrifuged at 15,000 rpm for 5min. Pellet was dissolved in 200µl of lysis buffer containing 0.03µg/µl of glycogen and 4µl of proteinase K (2 mg/ml). After incubation for 1h at 37°C, 300µl of NaI solution (6M NaI in 50mM Tris-HCl, 25mM EDTA, and pH 8.0 ± 0.2) and 500µl of isopropyl alcohol were aggregated to the suspension and then centrifuged at 10000g for 5min. The pellet was washed with 35% (v/v) of isopropyl alcohol, dried for 5min at 37°C, and then suspended in 20µl of sterilized water for PCR (21). The samples were then PCR tested.

Sensitivity of the extraction method from raw milk samples

0.5 ml of raw milk samples were artificially contaminated with 0.5ml of diluted samples of *L. monocytogenes;* the concentration of dilutions ranged between 10^1 and 10^9 CFU/ml; later on, the extraction procedure was executed as previously mentioned and 5µl (~200ng of DNA) of the extracted sample were taken for PCR assay following the conditions described before.

Reproducibility of PCR from raw milk samples

The PCR was made three times with each raw milk samples artificially contaminated with the smallest *L. monocytogenes* concentration (detection limit) that was detected in the sensitivity assay.

Statistical analysis

The results were analyzed in Epi-info 6.0d to observe the relevance of combining the Makino DNA extraction method and the Bansal modified PCR method (15,17,18,21). A previous test, composed of 10 repetitions, was used to guarantee measurement reliability; continuous variable agreement in the measures was estimated by using two measure systems. The statistic parameter used to estimate the agreement between two measures in the binary variable was the Kappa coefficient (K), which is defined as the agreement beyond chance divided by the possible agreement (22). A negative PCR (no amplification) or a non-specific amplification of sample DNA was considered as negative for *L. monocytogenes*; considerations for positive results were the amplification fragments of 938 and 750bp. The qualitative terms assigned to Kappa are the following: (0 - 0.2 = weak; 0.2 - 0.4 = good; 0.4 - 0.6 = moderate; 0.6 - 0.8 = substantial; 0.8 - 1 = almost perfect (22,23).

RESULTS AND DISCUSSION

Physical and chemical analysis of raw milk simples

For a microbial hazard, temperature is the major critical measure in several steps in dairy production; for this reason milk samples were aseptically taken and transported to the laboratory at 4°C, to avoid the multiplication of pathogens (24). Raw milk contains approximately 4% lactose, 3% protein and 3% fat, which could be used by several microorganisms as substrates for growth. In order to show the feasibility of the DNA extraction procedure and PCR method combination reported in this paper, it was essential to determine that raw milk samples were free of *L. monocytogenes* as well as testing that the physical and chemical characteristics were normal. Normal values of physical and chemical characteristics of the raw milk samples are summarized in Table 2, showing that normal physical and chemical characteristics do not interfere with the DNA extraction procedure or PCR (Figures 1 and 2).

Specificity of the PCR technique

The genomic DNA of 39 strains under study was tested in a PCR reaction. Results evidenced the

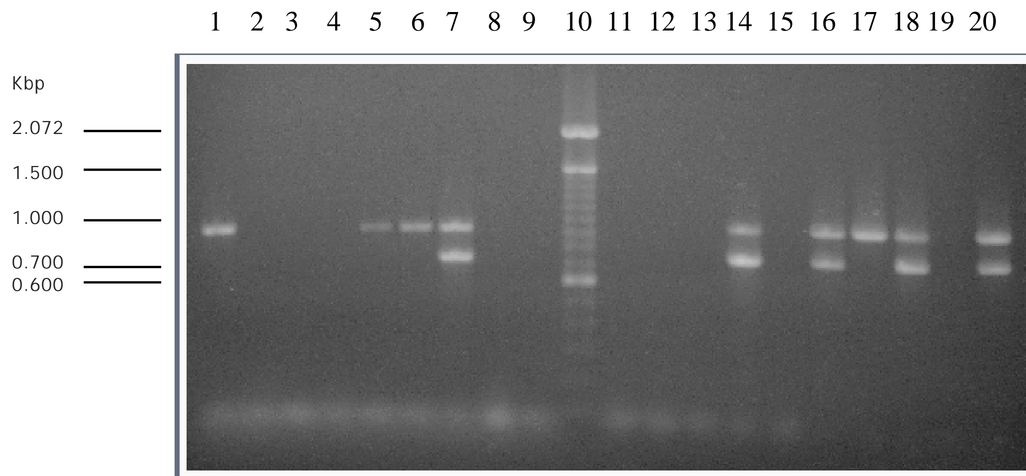

Figure 1. Specificity of PCR shown on a 1% (w/v) agarose gel electrophoresis. Well 1: DNA of *Listeria innocua*, well 2: DNA of *Staphylococcus aureus*, well 3: DNA of *Escherichia coli* O127, well 4: DNA of *Pseudomonas aeruginosa* ATCC 4027, well 5: DNA of *Listeria innocua* (Swiss), well 6: DNA of *Listeria innocua*, well 7: DNA of *Listeria monocytogenes* (from milk), well 8: DNA of *Salmonella enteritidis*, well 9: DNA of *Bacillus cereus*, well 10: Molecular size marker 100bp DNA Ladder (Invitrogen), well 11: White sample, well 12: DNA of *Lactococcus lactis*, well 13: DNA of *Proteus sp.*, well 14: DNA of *Listeria monocytogenes* ATCC 1915, well 15: DNA of *Enterococcus faecalis*, well 16: DNA of *Listeria monocytogenes* ATCC 4334, well 17: DNA of *Listeria innocua*, well 18: DNA of *Listeria monocytogenes* ATCC 4640, well 19: DNA of *Klebsiella spp.*, well 20: DNA of *Listeria monocytogenes* (from milk).

high specificity of the technique since all the strains of *L. monocytogenes* amplified the fragments 938bp and 750bp, specifically for genus and species, respectively (15,18,25). *L. innocua* strains only amplified the 938bp fragment, and took place due to the hybridization of the primers LU1 and U1 with the specific sequence of the coding region of the 16S rDNA of the *Listeria* genus. Negative amplification fragments were observed for other bacterial genera (Figure 1). In 1990, Border amplified *L.* monocytogenes DNA by PCR using 5 primers, two of them based on the sequences of the *hlyA* gene and other 3 primers based on the region 16S of the rDNA; however, the results showed fragment lengths of 702pb and 938pb (26). Allman (1995) used four primers, LO1, LO2, LO3 and LO4, to recognize the gene *hlyA* that produced fragment lengths of 234pb, 207pb and 204pb; this led to confirm PCR detection using the Nested PCRs technique (27). Moyra et al. (1996) used primers prfA and prfB with complementary sequences to the *prfA* gene, which is involved in the regulation of the listeriolysine synthesis (28). Both Allman (1995) and Moyra (1996) could only identify in their works the species, a fact that did not eliminate the interference of crossed reactions with other species of non pathogenic *Listeria* spp. (29).

Reproducibility of the PCR technique

The pilot test revealed that the technique is highly reproducible and statistically reliable. Since the analysis made by taking into account a kappa of a null hypothesis of 0.75 and alternative hypothesis of kappa 0.25 and a correct classification probability of 85%, generates a final Kappa of 0.85; according to some authors, this value is classified as "nearly perfect" in terms of agreement among the assayed variables (22,23).

Sensitivity of the PCR technique

Data showed an association of 95% in the reliability interval, a sensitivity of 100%, a specificity of 100%, a predictive positive value of 100%, and a predictive negative value of 100%. These results demonstrate that the detection of *Listeria* spp. is possible by using any of the three methods since they share the same sensitivity and specificity. One hundred percent of the predictive value for PCR (alternative method) provides high reliability, and

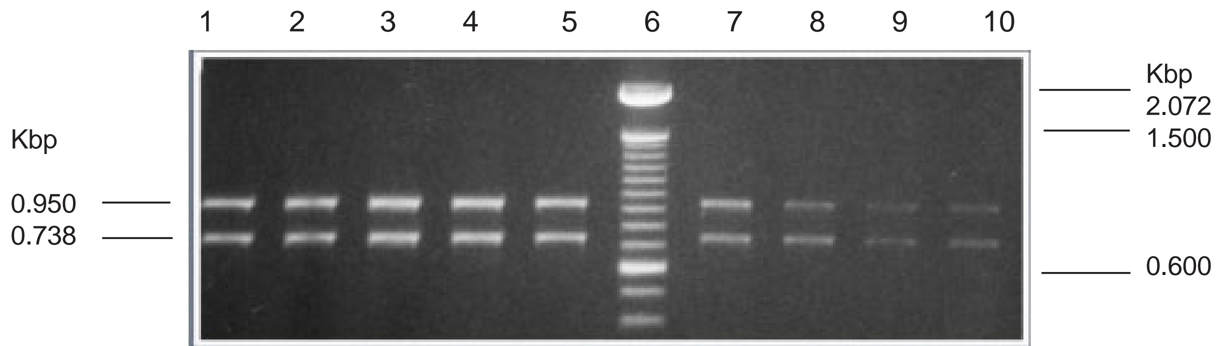

Figure 2. Sensitivity of the extraction technique starting from artificially contaminated raw milk samples. Results are shown on a 1% (w/v) agarose gel electrophoresis. Different CFU/ml of *Listeria monocytogenes* Well 1: 10^9 CFU/ml, *well 2: 10^8 CFU/ml, well 3: 10^7, well 4: 10^6 CFU/ml, well 5: 10^5 CFU/ml, well 6:* Molecular size marker 100bp DNA Ladder (Invitrogen), *well 7: 10^4 CFU/ml, well 8: 10^3 CFU/ml, well 9: 10^2 CFU/ml, well 10: 10^1 CFU/ml.*

Table 2. *Results of microbiological, physical and chemical analysis of raw milk samples before the artificial contamination with Listeria monocytogenes*

Sample No.	Alcohol test	pH	% Lactic acid	Fat Content (% w/v)
11	acid	6.0	0.85	3.70
13	normal	6.8	0.22	3.34
26	normal	6.8	0.25	3.64
30	normal	6.6	0.48	3.94
35	normal	6.8	0.25	3.79
38	normal	6.9	0.20	4.00
41	acid	6.8	0.24	3.62
46	normal	6.8	0.24	3.27
56	normal	6.9	0.16	3.48
59	normal	6.8	0.23	3.90
62	normal	6.8	0.20	3.61
9	normal	6.9	0.30	3.90
18	normal	6.6	0.48	3.72
20	normal	6.7	0.22	3.84
34	normal	6.7	0.22	3.71
39	normal	6.8	0.20	3.78
47	normal	6.7	0.22	3.62
48	normal	6.5	0.50	3.46
54	normal	6.6	0.48	3.72
58	normal	6.6	0.47	3.83

L. monocytogenes detection were negative; reductase, sucrose, chloride and starch tests were negative.

allows the detection of the positive samples that really contain the analyze, discarding negative samples with 100% of reliability. All artificially contaminated samples (with *L. monocytogenes*) amplified fragments of 938pb and 750pb; in the case of *E. coli* and *S. aureus,* these did not amplify any DNA fragments. The immunology method only permitted the detection of the *Listeria* genus, (following manufacturer's recommendations), and complementary tests were required to identify the species (data not shown). Nevertheless, the three methods showed similar results. In the food industry, however, a decisive factor is the time invested in quality tests. The traditional technique reported presumptive results in the first 5 days, and final results in 15 days; the immunology test provided results in 48h without discriminating between the *Listeria* species, while the PCR generated highly reliable, sensitive and reproducible results in 4 hours (30). The DNA extraction method from milk using NaI allowed bacterial DNA to be recovering. The detection limit of the PCR was 10^1 CFU/ml; subsequent treatments were not necessary for extraction to eliminate the interference caused by food components (Figures 2). The traditional method (Gold Standard) reports a detection limit above 10^2 CFU/ml. In the case of the immunology method, microorganism populations higher than 10^5 CFU/ml were required.

Finally, the PCR technique used in this study was highly reproducible and statistically reliable, showing a kappa of 0.85 in the level of agreement among assayed variables. The major finding of this paper was the possibility of reproducing Bansal PCR (17) and Makino DNA extraction (21) procedures, adapted to our conditions, and of validating the PCR technique for *L. monocytogenes* detection in raw milk samples. In recent years *L. monocytogenes* has been detected in several dairy products (31,32). Many Latin American countries have reported *L. monocytogenes* as food-borne emerging microorganism, causing abortions, sepsis and meningitis among other clinical signals leading to death (33,36). Taking into account that several foods appeared as vehicle of infections, we proposed an extraction procedure combined with a PCR method that reduced the time of identification of *L. monocytogenes* in raw milk, from 15 days (gold standard method) to ~5h. This PCR technique could be adapted and validated to be used for other types of food, such as cheeses, poultry and meat products. Moreover, the proposed technique could also be used in body fluids such as Cerebral Spinal Fluid (CSF) with the aim of rapid and reliable detection of the pathogen, which would be an important diagnostic tool to make prompt decisions for convenient treatment of patients.

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