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Direct detection of *Listeria monocytogenes* from milk by magnetic based DNA isolation and PCR

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Abstract

Two nucleic acid-based methods for rapid and sensitive detection of the foodborne pathogen *Listeria monocytogenes* in milk were developed in this study. These methods rely on paramagnetic nanoparticle-based isolation of bacterial DNA directly from milk and subsequent PCR with selective primers for the listeriolysin O (*hlyA*) gene. The *hlyA* specific product was reproducibly detected and showed a sensitivity of 10 cfu ml^{-1}. The magnetic-based system had a sensitivity 10-fold higher than that of commercially available column devices. The detection limit of both methods is sufficient for direct detection of *L. monocytogenes* DNA in milk avoiding the enrichment culturing step, reducing the time necessary to obtain results from samples to 7 h rather than the 5-day minimum required for the standard procedure. The methods developed are suitable for automation.

Keywords: *Listeria monocytogenes*; *hlyA*; Paramagnetic nanoparticles; Milk

1. Introduction

Listeria monocytogenes has been recognized as a cause of disease in humans and animals and has been responsible for listeriosis outbreaks in past years (Bille, 1990; Broome et al., 1990). Various reports show that *Listeria* spp. can be found in dairy products (Marth and Ryser, 1990), meat and poultry (Carosella, 1990), as well as in vegetables (Beuchat et al., 1990). Furthermore, several outbreaks of listeriosis were proven to be associated with the consumption of milk and are causing great concern in the dairy industry due to the number of cases and the nearly 30% overall mortality rate of these outbreaks (Griffiths, 1989). Special risk groups are pregnant women, newborns, immunocompromized patients and the elderly (Schuchat et al., 1991).

Current microbiological culture methods require a minimum of 5 days to isolate *Listeria* colonies from food matrices and additional days for the identification at the species level, and are therefore time-consuming and laborious (Rapporti ISTISAN, 1996). Thus, the development of rapid and sensitive methods for the detection of these pathogens in food are needed to overcome these limitations.

PCR-based detection systems, which are specific and sensitive, have been proposed to eliminate the enrichment culturing step (Makino et al., 1995; Lantz et al., 1994). However, application of PCR for the direct detection of pathogens present in foods has been limited by the complex composition of the starting materials, which contain inhibitors for PCR amplification (Rossen et al., 1992; Bickley et al., 1996). Moreover, when PCR was applied to milk samples, its sensitivity was low when compared to that of bacterial cultures, and some false-negative PCR results were reported (Romero et al., 1995), particularly at weak pathogen concentrations.

The aim of this study was to develop a rapid and sensitive method for *L. monocytogenes* detection in milk eliminating the need for enrichment and subsequent culturing on plating media.

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For this purpose, a protocol based on magnetic nanoparticles DNA isolation followed by PCR was developed. Two different magnetisable supports were used: silica-magnetite beads (Taylor et al., 2000), and agarose-based beads containing a paramagnetic component and derivatized with diethylaminoethyl (DEAE-Agarose) (Bruce et al., 1996). The results obtained with magnetic supports were compared to two other currently used methods: conventional phenol–chloroform extraction and commercial column devices.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and media

The bacterial species used in this study are as follows: *L. monocytogenes* (33 strains, one of which is ATCC 9525), *L. innocua* (6 strains), *Staphylococcus aureus* (1 strain), originally isolated from foods, and *Salmonella abortus ovis* (1 strain) from a clinical case from an infected animal kindly provided by the "Istituto Zooprofilattico Umbria e Marche (Perugia, Italy)"; *Enterococcus faecalis* (2 strains), *Escherichia coli* henterotoxic HB LT+ (2 strains), *Salmonella* spp. (1 strain) and *Streptococcus β-haemoliticus* (1 strain) isolated from a nosocomial environment.

L. monocytogenes was cultured in listeria enrichment broth base (LEB) with nalidixic acid ($40\,mg\,l^{-1}$), cycloheximide ($50\,mg\,l^{-1}$) and listeria selective agar base (Oxford) for 24 h at 37°C. For *L. monocytogenes* detection and enumeration in milk samples microbiological standard methods were used (Rapporti ISTISAN, 1996; Ord. Min. San. 7/12/1993, all. 3).

For the routine culture of the other bacterial species, tryptone soya agar (TSA) was used. All culture media were from Oxoid (Basingstoke, England).

Pasteurized whole bovine milk was obtained from a local supermarket.

2.2. Primer selection

Two oligonucleotide primers were designed using Oligo Primer Analysis software, version 6 (MedProbe, Oslo, Norway), according to the determined sequence of the listeriolysin O gene (*hlyA*) (GenBank accession no. AF253320). The sequences, designated LL7 and LL8, are those reported in Table 1. The primers were purchased from Sigma (Sigma-Genosys, Cambridge, UK).

2.2.1. Target construction

Construction of the plasmid encoding a 702 bp sequence of the *L. monocytogenes hlyA* gene (145–846 GenBank accession no. AF253320) was as follows: *L. monocytogenes* total DNA was used as a template in PCR (GeneAmp PCR System 2400, Applied Biosystems, Foster City, CA) with LM1-LM2 primers (Sigma-Genosys), as described by Herman et al. (1995). Primers sequences are shown in Table 1. The amplified DNA segment was cloned into the pCR 2.1 vector (Invitrogen, Groningen, NL). The new plasmid was then designated p702. The recombinant plasmid was introduced into *E. coli* INVα′ (Invitrogen).

2.3. Primer validation

2.3.1. Primer sensitivity test

The DNA template for the sensitivity test was obtained by extracting p702 plasmid using a commercial kit (Qiagen Plasmid Midi Kit, Qiagen GmbH, Germany). The plasmid was linearized with *BamH*I and its amount was determined by agarose gel analysis. Tenfold serial dilutions of p702 in H_2O milliQ were made, and 10^9–1 molecule per PCR were amplified with LL7–LL8 primers as described below. Amplicons were visualized by gel electrophoresis to determine the sensitivity of the PCR assay.

2.3.2. Primer specificity tests

Bacterial species screened for the primers specificity tests are listed in the Section 2.1 above. All the *L. monocytogenes* strains used for specificity tests have been previously characterized on the basis of lineage, according to Jinneman and Hill (2001).

Genomic DNA was obtained from typical colonies by lysing bacterial cells at 100°C for 10 min. The cellular debris was pelleted by centrifugation at 12000*g* for 10 min and 5 μl of the clear supernatant fluid containing nucleic acids were used for subsequent PCR reactions with LL7–LL8 primers.

2.4. PCR assay

PCR amplification was performed in a total reaction volume of 50 μl, with HotStarTaq DNA Polymerase kit (Qiagen). The reaction mixture contained 3.5 mM $MgCl_2$, 150 μM of each deoxynucleoside triphosphate (Applied Biosystems), 25 pmol of each primer and 1 U *Taq* DNA Polymerase. The amplification profile was as follows: 95°C for 15 min; 50 cycles of 95°C for 15 s, 61°C for 20 s, 72°C for 30 s; final extension at 72°C for 4 min. Reactions were thermally cycled in a GeneAmp PCR System 2400.

PCR products were detected by gel electrophoresis in 1.8% agarose gel (Sigma, St. Louis, MI) stained with ethidium bromide ($0.5\,\mu g\,ml^{-1}$). Molecular weight size markers (Marker VI (Roche) and ΦX174 DNA/*BsuR*I (*Hae*III) (MBI Fermentas)) were included in each gel.

Table 1
Nucleotide sequence of oligonucleotide primers used in this study

Primer	Orientation	Sequence (5′→3′)	G+C content (%)	Length	T_m	Location within gene (bp)[a]
LL7	Forward	TTG CCA GGA ATG ACT AAT CAA G	40.9	22	62.8	472–493
LL8	Reverse	ATT CAC TGT AAG CCA TTT CGT C	40.9	22	61.8	622–643
LM1[b]	Forward	CCT AAG ACG CCA ATC GAA	50	18	61.3	145–162
LM2[b]	Reverse	AAG CGC TTG CAA CTG CTC	55.5	18	64.5	829–846

[a] From published *hlyA* gene cds for listeriolysin O (GenBank accession no. AF253320).
[b] Herman et al. (1995).

2.5. Detection of L. monocytogenes in milk

2.5.1. Sample contamination

For milk contamination, a *L. monocytogenes* strain (ATCC 9525) was employed. Pasteurized whole milk was artificially contaminated with a bacterial culture, having an optical density (OD) of 0.065 (at 600 nm) corresponding to 10^8 cfu ml^{-1}, by adding 1 ml 10-fold dilutions in milk samples of 9 ml. The bacterial number in the culture was subsequently estimated by standard plate counts.

Bacterial cells in milk samples were recovered by centrifugation at 6000*g* for 20 min at 4°C and the pellets were then subjected to DNA extraction, as follows.

2.5.2. DEAE-Agarose extraction protocol

DEAE-Agarose was produced as previously reported (Bruce et al., 1996). Prior to use, a 4% suspension of DEAE-Agarose (150 μl) in storage buffer (20% aqueous methanol) was placed in a 1.5 ml microcentrifuge tube and the beads immobilized using a magnetic stand (Promega, Madison, WI). The supernatant was removed and the beads washed 4 times with 1 ml of sterile distilled water, once with 150 μl of STET buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% (w/v) Triton-X-100, 100 mM NaCl) and once again with 1 ml of sterile distilled water.

The bacterial pellets were resuspended with 450 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% (w/v) Triton-X-100). Nine microliters of 10 mg ml^{-1} RNase A (Sigma) and 40 μl of 20 mg ml^{-1} Proteinase K (Sigma) were added. After incubation at 37°C for 30 min, the samples were gently mixed with 150 μl of DEAE-Agarose for 5 min at room temperature. The beads were again immobilized and the supernatant removed. The beads were washed by resuspension in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 400 mM NaCl (400 μl). After immobilization of the beads, the supernatant was discarded and the DNA desorbed by addition of 50 mM Arginine free base (ICN, Irvine, CA) in NaCl 1 M (200 μl), incubating for 5 min at 65°C. The beads were immobilized and the supernatant transferred to a clean tube. The elution step was repeated and the eluates combined.

2.5.3. Silica magnetite extraction protocol

For this extraction, silica-magnetite-based solid-phase supports, produced as previously reported (Taylor et al., 2000), were used.

Prior to use, the beads were washed twice with 1 ml of sterile H_2O to remove storage buffer and dispensed in 50 μl (2 mg) aliquots in sterile 1.5 ml microcentrifuge tubes.

The bacterial pellets were resuspended in 20 mM of Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% (w/v) Triton-X-100, 20 mg ml^{-1} lysozyme (Sigma) (450 μl) and incubated at 37°C for 30 min. Subsequently, 9 μl of 10 mg ml^{-1} RNase A were added and the lysates were incubated for 10 min at room temperature. The samples were digested with 40 μl of 20 mg ml^{-1} Proteinase K for 1 h at 55°C, then transferred to the bead-containing tube and 600 μl of 20% polyethyleneglycol 8000 (Sigma) in 4 M NaCl were added. The mixtures were incubated with end over end rotation at room temperature for 15 min, in order to allow DNA to adsorb to the beads. The beads were immobilized using the magnetic stand, supernatants were removed and discarded and the beads washed in 200 μl of 50% ethanol for 5 min at room temperature. The beads were magnetically immobilized, supernatants were removed and discarded, and the beads left to air dry for 2 min. DNA was eluted from the beads by the addition of 200 μl of nuclease-free water and incubation for 10 min at 65°C. The elution step was repeated twice and the eluates combined.

2.5.4. Phenol–chloroform extraction followed by silica magnetite purification

For DNA isolation, bacterial pellets were subjected to phenol–chloroform extraction according to the standard method (Sambrook et al., 1989). Briefly, an equal volume of phenol:chloroform:isoamyl alcool (25:24:1) was added to the sample, mixed and centrifuged at 12,000*g* for 6 min. An equal volume of chloroform:isoamyl alcool (24:1) was added to the aqueous phase and the sample was mixed and centrifuged as above. The aqueous phase obtained was added to 300 μl of 4 M guanidine thiocyanate and the samples were processed as described by silica magnetite extraction protocol.

2.5.5. Phenol–chloroform extraction protocol

The bacterial pellets obtained in Section 2.5.1 were subjected to phenol–chloroform extraction according to the standard method (Sambrook et al., 1989) described in Section 2.5.4.

2.5.6. DNeasy tissue kit qiagen

The extraction was performed according to manufacturer instructions for Gram-positive bacteria.

2.5.7. Amplification of DNA extracted from milk

DNA in all the eluates was precipitated according to standard methods (Sambrook et al., 1989). Briefly, 2.5 volumes of ethanol and 1/10 volume of 0.3 M sodium acetate pH 5.2 were added to the sample. After 30 min at −20°C, the sample was centrifuged at 15,000*g* for 15 min, washed with 50 µl of 70% ethanol, centrifuging for 5 min as above, and resuspended with 20 µl of nuclease-free water. Ten microliters of the extracted DNA were used for PCR assay.

As a positive control, 5 µl of *L. monocytogenes* ATCC 9525 DNA, obtained by lysis at 100°C, were included.

PCR and PCR product detection by gel electrophoresis were performed as described in Section 2.4.

3. Results

3.1. PCR specificity

Six *Listeria* species are known to exist. In an attempt to identify *L. monocytogenes* by PCR-based detection, potentially suitable oligonucleotide primer sequences complementary to the virulence gene *hlyA* (GenBank accession no. AF253320) and known to be specific for *L. monocytogenes*, were examined for specificity using basic local alignment search tool (BLAST, www.ncbi.nlm.nih.gov/BLAST/). Results showed that primers were specific for *L. monocytogenes* and that this specificity was constant among different strains of this species.

The primers LL7 and LL8 were selected to amplify a 172 bp region of the *hlyA* gene. On the basis of the published nucleotide sequence reported by Giammarini et al. (2003), primer location within the coding region of the listeriolysin O gene and sequences are those reported in Table 1.

To confirm the specificity of these primers for *L. monocytogenes*, PCR amplification was performed on DNA obtained from 33 isolates of *L. monocytogenes*, of which 7 strains (21%) of Type 1, 25 (76%) of Type 2 and 1 (3%) of Type 3 (ATCC 9525 strain), as well as other bacterial species.

The PCR amplification using LL7 and LL8 primers generated a product of expected size from all *L. monocytogenes* strains tested but not from *L. innocua* strains and other bacterial species, including haemolysin-producing bacteria (Fig. 1). Moreover, unspecific amplicons were not detected in any of the species tested. These results clearly indicate that the primers LL7 and LL8 have a high affinity for the correct target sequence and are specific for *L. monocytogenes*.

3.2. PCR sensitivity

The sensitivity of the PCR was determined by using 10-fold serial dilutions of a known amount of a recombinant plasmid (p702) containing an insert target sequence of the *hlyA* gene. The PCR product was detected with as few as 10 molecules of this plasmid per PCR (Fig. 2). Since *hlyA* is a single copy gene within the *L. monocytogenes* genome (Mengaud et al., 1988), this outcome corresponds to a detection limit of 10 bacterial cells.

3.3. Detection of L. monocytogenes in milk

In order to assess the sensitivity of the selected extraction methods in milk, 10 ml samples were artificially contaminated with different concentrations of *L. monocytogenes*. The contamination ranged from 10^5 to 1 cfu ml^{-1} of milk. Centrifugation of the sample before extraction was considered necessary for bacterial concentration and to eliminate milk components in the supernatant known to be PCR inhibitors (Rossen et al.,

Fig. 1. Specificity of the PCR assay. Lanes: M, Marker VI (Roche): (1) *E. faecalis* 4421; (2) *E. faecalis* 4463; (3) *E. coli* HB LT + 68; (4) *E. coli* HB LT + 100; (5) *Salmonella* spp.; (6) *Salmonella abortus ovis*; (7) *S. aureus*; (8) *Streptococcus β haemoliticus*; (9) *L. innocua*; (10) *L. monocytogenes*; and (11) PCR negative control (no DNA).

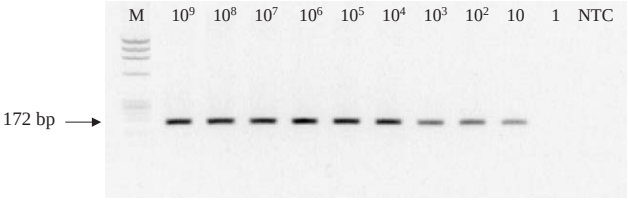

Fig. 2. Sensitivity of the PCR assay with p702 plasmid 10-fold serial dilutions from 10^9 to 1 molecule. Lanes: M, ΦX174 DNA/*Bsu*RI (*Hae*III) marker (MBI Fermentas); NTC, PCR negative control (no DNA).

Table 2
Detection limits of various extraction methods employed for *L. monocytogenes* detection in milk

	L. monocytogenes (cfu ml^{-1}) in milk					
	10^5	10^4	10^3	10^2	10	1
Phenol–chloroform	+	+	+	+	+	–
Phenol–chloroform + silica magnetite	+	+	+	+	+	–
Silica magnetite	+	+	+	+	+	–
Dneasy tissue kit (Qiagen)	+	+	+	+	–	–
DEAE-Agarose	+	+	+	+	+	±[a]

[a] The contamination was detected in two of 20 examined samples.

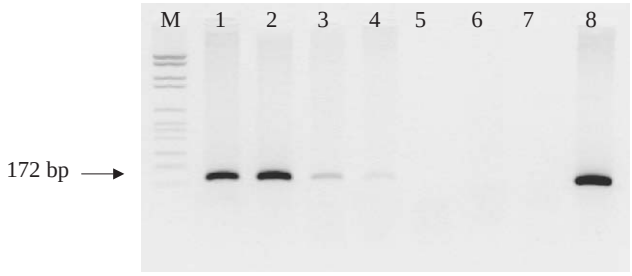

Fig. 3. Agarose gel electrophoresis of the amplification products of *L. monocytogenes* DNA obtained through different extraction protocols from 10 ml milk samples artificially contaminated with 10 cfu ml^{-1}. Lanes: M, Marker VI (Roche): (1) DEAE-Agarose extraction; (2) silica magnetite extraction; (3) Phenol–chloroform extraction followed by silica magnetite purification; (4) Phenol–chloroform extraction; (5) DNeasy Tissue kit (Qiagen) extraction; (6) blank (non-contaminated milk); (7) PCR negative control (no DNA); and (8) PCR positive control (*L. monocytogenes* DNA).

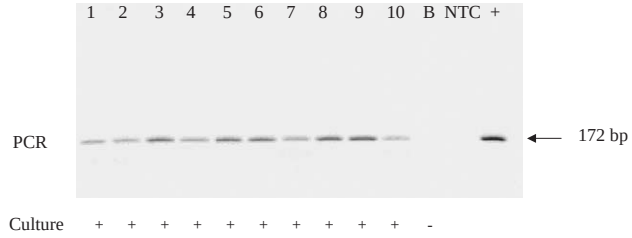

Fig. 4. Comparison of *L. monocytogenes* detection efficiency between the microbiological standard method and DEAE-Agarose extraction followed by PCR. Lanes 1–10: 10 ml milk samples artificially contaminated with 10 cfu ml^{-1}; B—blank (non-contaminated milk); NTC—PCR negative control (no DNA); and +—PCR positive control (*L. monocytogenes* DNA).

1992; Bickley et al., 1996). The extraction methods were compared on the basis of amplificability of the DNA extracted. Since pathogen DNA represents only a small fraction of total sample DNA extracted, we did not assess nucleic acid concentrations in the samples before PCR.

Detection limits for each method is listed in Table 2. The highest sensitivity, corresponding to 10 cfu ml^{-1}, was achieved for samples extracted using magnetic supports, phenol–chloroform and phenol–chloroform plus silica magnetite. However, the highest signals were obtained using magnetic-based methods (Fig. 3). The sensitivity obtained with DEAE-Agarose proved to be reproducible when the test was repeated with several samples, all contaminated with 10 cfu ml^{-1} (Figs. 4 and 5). Moreover, in two experiments we were also able to detect a contamination level as low as 1 cfu ml^{-1}. For control purposes, uninoculated samples of milk were also subjected to all sample preparation methods and to PCR. No amplification was observed (Figs. 3 and 4).

In order to compare the DEAE-Agarose-based detection method to the standard culture procedure, 50% of 20 milk samples were randomly contaminated with 10 cfu ml^{-1} of *L. monocytogenes* and subsequently analysed. The results indicated that both the procedures correctly identified the artificially contaminated milk samples and were in perfect agreement, with no false positives or false negatives (Fig. 4).

In Fig. 5 are shown the percentages of positive *L. monocytogenes* contaminated milk samples as a function of bacterial cfu. Ten milliliters of milk samples were contaminated with serial dilutions of *L. monocytogenes* and then processed for bacterial DNA detection with DEAE-Agarose. Contamination levels (cfu ml^{-1}) recovered in milk were plotted versus percentages of positive samples. Data are the mean of three different experiments, each performed with 10 samples for each contamination rate. All samples containing 10 cfu ml^{-1} or more were always detected as positive.

4. Discussion

The detection of pathogenic bacteria is a fundamental objective of food microbiology ensuring food quality. The current microbiological culture procedures are laborious and time-consuming. Consequently, there is an increased need for a rapid and reliable detection method to guarantee food safety. Regarding this, PCR technology has successfully shortened analysis time and has been widely applied for the detection of food-borne pathogens (Bruce, 1994). Several of these PCR-based methods were developed for the detection of

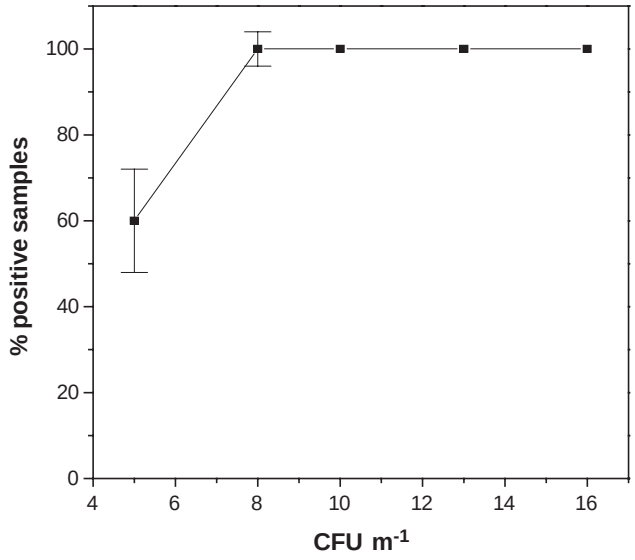

Fig. 5. Percentage of positive *L. monocytogenes* contaminated milk samples detected as a function of bacterial CFU. Contamination levels (cfu ml^{-1}) recovered in milk were plotted versus percentages of positive samples. Data are the mean of three different experiments, each performed with 10 samples for each contamination rate.

L. monocytogenes and involve a preenrichment step (Bansal, 1996; Manzano et al., 1997), the use of monoclonal antibody-coated beads for bacteria concentration (Fluit et al., 1993; Nogva et al., 2000) or the chemical extraction of food components (Herman et al., 1995).

In this study, the development of two magnetic-based methods for rapid and direct detection of *L. monocytogenes* in milk is reported. A new set of primers targeting the *hlyA* gene has been designed because preliminary experiments using other sequences (Herman et al., 1995) showed less sensitivity and specificity. It should be noticed, however, that, differently from these Authors, we did not perform a nested PCR because this method extends analysis time and is considered susceptible to contamination risks (Scheu et al., 1998). Primers specificity was assessed with *L. monocytogenes* isolates belonging to each of the three evolutionary lineages defined by Jinneman and Hill (2001).

The properties of the magnetic supports (silica magnetite beads and DEAE-Agarose) and their suitability for batchwise purification of nucleic acids, have been previously reported (Taylor et al., 2000; Levison et al., 1998; Bruce et al, 1996). The use of magnetic nanoparticles for sample purification proved necessary to obtain good quality DNA and, above all, free of other contaminant molecules of food origin, such as proteins, fats or calcium ions, well known to be PCR inhibitors (Rossen et al., 1992; Bickley et al., 1996).

Both procedures showed an elevated level of sensitivity and specificity, with a detection level corresponding to 10 cfu ml^{-1}. Comparable results were also reported by Herman et al. (1995) utilizing a nested PCR; however, nested PCR is considered not suitable to diagnostic, because, as discussed above, it increases the risk of detecting contaminating PCR-products (Scheu et al., 1998).

Similar sensitivity was also obtained with the phenol–chloroform procedure, with or without the additional step of purification using silica beads. Procedures involving the use of organic solvents were also proposed by other authors (Choi and Hong, 2003; Koo and Jaykus, 2003; Corrente et al., 2001; Herman et al., 1995; Starbuck et al., 1992), however, these methods, even though showed good sensitivity, presents the disadvantage of toxic reagents and the manipulation of organic solvents. In contrast, protocols without organic solvents resulted less sensitive (Hein et al., 2001).

Finally, the commercial system tested was less sensitive (detection limit 10^2 cfu ml^{-1}). However, this system is not specifically suggested for detection in foods. The time required for magnetic extraction of bacterial DNA, PCR and gel electrophoresis is approximately 1 working day. Thus, this detection method is considerably less time-consuming than the classic procedures used for *L. monocytogenes* detection, requiring at least 1 week. Moreover, our method is safe and easy to perform since it does not involve the phenol–chloroform extraction procedure. Furthermore, since the preenrichment step is avoided, it could be applied to the semi-quantitative estimation of the number of bacterial cells present in milk samples when the PCR described here will be substituted with a competitive PCR or real-time PCR system. For this purpose, experiments are in progress.

The magnetically based procedure may present the additional advantage of being suitable for automation systems, minimizing manual labour and time while providing more reproducible results and being useful for large-scale analyses.

Since DNA-based PCR so far cannot distinguish between DNA of live or dead bacteria, the method presented could be used as a screening method of unprocessed milk samples and should be further evaluated by examining field samples and compare the results of the microbiological method and the PCR methods.

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