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ORIGINAL PAPER

Development of a nucleic acid lateral flow immunoassay for simultaneous detection of *Listeria* spp. and *Listeria monocytogenes* in food

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Abstract We present a new nucleic acid lateral flow immunoassay (NALFIA) for the assessment of listeria contamination. The detection procedure starts with enrichment of sample in Half Fraser broth (24 h). Following isolation of DNA, a duplex PCR is performed with two labelled primer sets, one generic and directed to a specific sequence of the gene encoding 16S rRNA from *Listeria* spp. and the other specific and directed to a part of the *prfA* gene encoding the central virulence gene regulator from the food pathogen *Listeria monocytogenes* (3.5 h). The PCR solution is directly added to the one-step assay device and the appearance of a grey/black line is indicative of the presence of specific amplicons (max 15 min). In all tests performed, the method correctly identified *L. monocytogenes* and strains of *Listeria* spp. PCR material of over 20 food samples was tested by NALFIA. The method proved to be useful for the detection of *L. monocytogenes* in different kinds of food samples.

Keywords Food-borne pathogen · Immunochromatographic test · Nucleic acid lateral flow immunoassay (NALFIA) · *Listeria monocytogenes* · Food · Nucleic acid testing · Nanoparticles

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Introduction

Each year, millions of people become ill from food-borne diseases caused by *Salmonella*, *Listeria*, and *Campylobacter* [1]. For example, only in EU in 2005, it was reported in nearly 400,000 cases [2]. The effective control of food safety can be improved by the detection of pathogenic microorganisms with rapid methods. These should be simple, cost effective, easy to interpret, and with sufficient sensitivity and specificity.

The genus *Listeria* comprises six recognized species: *L. monocytogenes*, *L. ivanovii* ssp. *ivanovii* and *londoniensis*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*. *Listeria* can survive and grow over a wide range of environmental conditions [3]. This allows the listeria to overcome food preservation and safety barriers [4]. Although occurrence of *Listeria* strains in food may indicate errors in good hygienic and manufacturing practice, only *L. monocytogenes* is a significant human and animal pathogen, which is responsible for severe invasive disease, listeriosis [5, 6].

Outbreaks caused by *L. monocytogenes* have been associated with ingestion of a variety of contaminated foods and were reported from many countries, including Australia, Switzerland, France, and the United States [7–10].

The minimum infective dose of *L. monocytogenes* has not been established. According to the Commission Regulation No 1444/2007, the food law of European Union insists on the absence of *L. monocytogenes* (not detectable in 25 g of food product) in ready-to-eat foods intended for infants and for special medical purposes. The ready-to-eat foods, other than those mentioned before, may contain <100 cfu/g during their shelf life [11]. The standard isolation method for *L. monocytogenes* from food samples is described in ISO 11290-1 and 11290-2 [12]. Despite recent changes in ISO procedure, when application of modern

chromogenic medium (Agar Listeria Ottavani & Agosti) was included [12, 13], the total procedure takes 5–7 days, which is not optimal for testing foods with short shelf lives.

With the goal to overcome problems associated with traditional microbiological methods such as being time consuming and labour intensive many new modern techniques have been developed. They are based on either chromogenic media [14–16], antibodies [17–19], or nucleic acid-based techniques [20, 21]. Identification of *Listeria* spp. and *L. monocytogenes* using nucleic acid testing is becoming increasingly popular. The major advantage of these nucleic acid-based techniques is the fact that specificity is based on genomic sequences and does not rely on the expression of particular antigens or enzymes. In addition, nucleic acid-based methods can be extremely sensitive and specific. A number of test kits for *Listeria* are commercially available [22, 23].

The aim of this study was to develop a rapid and sensitive nucleic acid method for detection of *L. monocytogenes* in particular and the genus *Listeria* in general, with a substantially reduced pre-enrichment phase. For the detection of amplicons, we used a one-step nucleic acid lateral flow immunoassay (NALFIA) [24]. In this method, a small volume of the final PCR solution is directly added to the one-step assay device and the appearance of a grey/black line is indicative of the presence of the specific amplicon. The visualization is mediated by using carbon nanoparticles. In the past years, nanoparticles were introduced as a detection tool in bioanalytics [25], which gives an alternative to conventional enzymatic or fluorescent labels, and enables easy and clear interpretation.

Materials and methods

Cultures

Strains of microorganisms used in the experiments are shown in Table 1. Cultures of *Listeria* and other bacteria were maintained on Petri plates of Oxford agar (Oxoid) or Brain Hearth Infusion Agar (Oxoid), respectively at 4 °C. Prior to experiments, cultures were grown in 5 ml Fraser Broth Base (Oxoid) at 37 °C for 18 h.

Sample preparation

Model milk samples for verification of NALFIA were artificially contaminated with four different strains of *L. monocytogenes* (microbiological reference strain ALM 92 was tested in triplicate, *L. monocytogenes* NCTC 4886, *L. monocytogenes* NCTC 4885, and *L. monocytogenes* isolated from milk in the Institute of Food Research, Norwich, UK), two other *Listeria* subspecies (*L. innocua*, *L. ivanovii*), and *Enterococcus faecalis*. Decimal dilutions of overnight cultures of all tested bacterial strains were prepared. One ml aliquots of the cultures containing less than 10 cells were inoculated into flasks containing 25 ml of sterilized milk. One non-contaminated milk sample was cultivated in the same way as a negative control.

The developed NALFIA was applied on real food samples which were prepared by the following procedure. Twelve food samples were collected from two supermarket chains, seven samples were obtained from one milk-producing biofarm in the Czech Republic, and five samples were part of internal lab trials.

All samples were cultivated according to the present standard isolation method for *L. monocytogenes* from food samples [12]. Flasks containing 225 ml of Half Fraser broth (Oxoid) were mixed with 25 ml or 25 g of food and incubated for 24 h at 37 °C whilst shaking. After this procedure, samples (1 ml) were used for DNA isolation. The results were confirmed by the standard isolation microbiological method with confirmation on chromogenic agar ALOA (Agar Listeria according to Ottaviani and Agosti, Bio-rad). Total bacterial counts in solutions used in this study were determined by plating on BHI agar (Oxoid). The plates were incubated at 37 °C for 48 h.

DNA extraction

The DNA extractions from enriched samples were performed using a GenElute Bacterial Genomic DNA kit (Sigma), with the Gram-positive bacterial protocol.

Amplification of genetic material

A specific sequence (1,003 bp) of the gene encoding 16S rRNA from *Listeria* spp. was amplified by PCR using 5′-end labelled specific primers [26]. One was labelled with biotin (B, primer C: 5′-AGG TTG ACC CTA CCG ACT TC-3′) and the other with fluorescein (FL, primer D: 5′-CAA GGA TAA GAG TAA CTG C-3′). A set of primers [27] specific for *L. monocytogenes* was used to amplify a part (274 bp) of the *prfA* gene encoding the central virulence gene regulator from this food pathogen. One of these primers was labelled with digoxigenin (DIG, primer LIP2: 5′-GTG TAA TCT TGA TGC CAT CAG G-3′) and the other with biotin (primer LIP1: 5′-GAT ACA GAA ACA TCG GTT GGC-3′). The optimized composition of the reaction mixture was 2.5 µl of FastStart *Taq* DNA Polymerase buffer (500 mM Tris/HCl, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, pH 8.3), 2.5 mM $MgCl_2$, 0.15 mM of dNTP, 2 U of FastStart *Taq* DNA Polymerase (all from Roche), 0.1 µM of primers LIP1 and LIP2, 0.2 µM of primers C and D, and 2 µl of prepared template DNA. The final volume was adjusted to 25 µl with distilled water. The

Table 1 Strains of bacteria used in this study

Species/strain (serotype)	Origin	Species/strain	Origin
L. monocytogenes	Ref. material ALM 92, NCTC 13273	*Bacillus cereus*	AFSG
L. monocytogenes (1/2a)	NCTC 4886	*Enterobacter cloacae*	Cabbage at STU
L. monocytogenes (4b)	NCTC 4885	*Enterococcus faecalis*	CCM 2308
L. monocytogenes	Milk from IFR	*Lactobacillus plantarum*	CCM 7039
L. monocytogenes (1/2c)	NCTC 5348	*Enterobacter sakazakii*	ATCC 29544
L. monocytogenes (4a)	NCTC 5214	*E. sakazakii*	CCM 3460
L. monocytogenes (4b)	NCTC 10527	*Staphylococcus epidermidis*	ATCC 12228
L. monocytogenes	NCTC 10888	*Staphylococcus aureus* ssp. *aureus*	ATCC 25923
L. monocytogenes	Chicken from IFR	*Proteus* sp.	CCM 1799
L. innocua	Salad from IFR	*Escherichia coli*	ATCC 8739
L. ivanovii	PHLS	*Salmonella enterica* ssp. *enterica*, *enteritidis*	ATCC 13076
L. seeligeri	ATCC 35967	*S. enterica* ssp. *enterica*, *typhimurium*	ATCC 14028
L. grayi	ATCC 19120	*Serratia marcescens* ssp. *marcescens*	ATCC 13880
L. welshimeri	NCTC 11857	*Pseudomonas aeruginosa*	ATCC 27853

NCTC UK National Collection of Type Cultures, *ATCC* American Type Culture Collection, *PHLS* UK Public Health Laboratory Service, *IFR* Institute of Food Research, Norwich, *CCM* Czech Collection of Microorganisms, *AFSG* Agrotechnology & Food Sciences Group, The Netherlands, *STU* Slovak University of Technology, Slovak Republic

PCR was performed in the GeneAmp 9700 96-well thermal cycler (Applied Biosystems) with the following steps: an initial denaturation at 95 °C for 4 min, 25 cycles each having a denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 74 °C for 1 min with a final extension at 74 °C for 5 min.

Following PCR, two types of double-labelled amplicons could be obtained: amplicons labelled with digoxigenin on one side and biotin on the other side (DIG-labelled) if *Listeria monocytogenes* template DNA was present and amplicons labelled with fluorescein and biotin (FL-labelled) if *Listeria* spp. DNA was present.

Immune reagents

NeutrAvidin Biotin-Binding Protein was from Pierce Biotechnology (Rockford, IL, USA) and Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG (Biotin, 2000 ng IgG/5 mm) was from Jackson ImmunoResearch (Suffolk, UK). Affinity-purified goat anti-fluorescein antibody (anti-FL, 500 ng/5 mm) was obtained from Biomeda (Foster City, CA, USA) and polyclonal anti-digoxigenin antibody (anti-DIG, 200 ng/5 mm) was from Roche Diagnostics (Basel, Switzerland).

Nucleic acid lateral flow immunoassay

As an assay format for preliminary characterization, an experimental setup using the wells of a microtitrate plate was used. A tube format [28] was applied for final evaluation of the assay with food samples (Fig. 1).

Antibodies were immobilized on nitrocellulose membranes by spraying using a Linomat V (CAMAG, Muttenz, Switzerland). A control line (CL) was added by spraying Biotin-conjugated AffiniPure Goat Anti-Mouse IgG, a test line specific to all species from the genus *Listeria* (TL1) by spraying anti-FL, and a test line specific to *Listeria monocytogenes* (TL2) by spraying anti-DIG.

Following PCR, 3 µl of the reaction mixture containing the specific amplicons was mixed with 1 µl of carbon nanoparticles–NeutrAvidin conjugate, able to interact with the biotin-labelled amplicons, and running buffer (100 mM borate buffer, pH 8.8, 1% (w/v) BSA, 0.05% (v/v) Tween 20, 0.02% (w/v) NaN_3) to a total volume of 100 µl. The mixture was run through nitrocellulose membranes and the result was read after maximally 15 min. Typical NALFIA results are shown in Fig. 2, together with electrophoresis. Labelled amplicons from samples containing *L. monocytogenes* (Fl- and DIG-labelled) were demonstrated by the appearance of three grey/black lines. Samples with the *Listeria* spp. but not *L. monocyotogenes* contained FL-labelled amplicons and were indicated by the appearance of two grey/black lines. PCR product prepared from samples without listeria template DNA showed only one grey/black line (control line).

Sensitivity analysis of NALFIA

Dilution ranges of DIG- and FL-labelled amplicons were prepared and tested in NALFIA. Amplicon concentrations were determined by the spectrophotometer NanoDrop (ND 1000, NanoDrop Technologies). Test lines were scanned

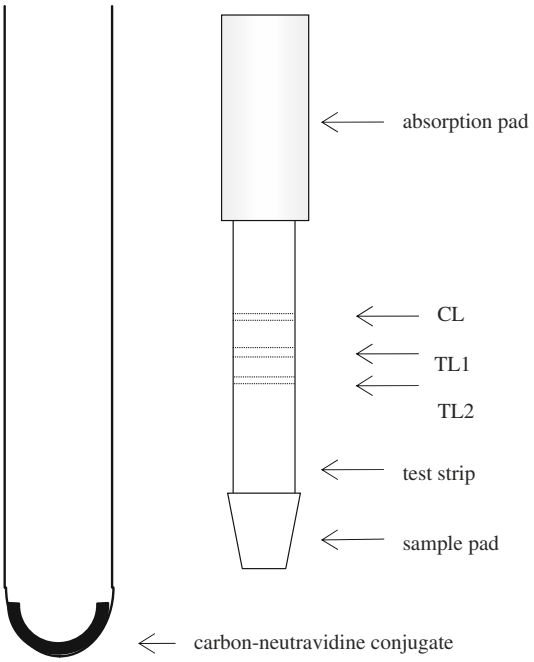

Fig. 1 A schematic drawing of a one-step tube assay [28]. Carbon–NeutrAvidin conjugate is dried on the bottom of the tube. Sample is applied on top of the conjugate and the strip is placed into the tube. If *L. monocytogenes* is present three grey/black lines will appear, if other *Listeria* species are present two lines will appear, and if the sample is negative only one control line will appear. *CL* control line formed by Biotin-conjugated AffiniPure Goat Anti-Mouse IgG, *TL1* test line specific to all species from the genus *Listeria* formed by α-FL, *TL2* test line specific to *Listeria monocytogenes* formed by α-DIG

with a flatbed scanner (Epson Perfection V700 Photo) and the line intensities were converted to pixel grey volumes with TotalLab (Nonlinear Dynamics, Ltd.). Curve fitting was done with Excel (Microsoft Office).

Results and discussion

Development of NALFIA for *Listeria* detection

We present a rapid, reliable, and specific immunochromatographic tube assay for simultaneous nucleic acid detection of *Listeria* spp. and *L. monocytogenes*.

The developed procedure consists of an enrichment step (24 h), isolation of template DNA and a PCR amplification step of specific sequences (3.5 h), and an immunochromatographic detection of amplified product by NALFIA (5–15 min). To our knowledge, this is the first time that this combination of PCR and immunochromatographic one-step detection is described for *Listeria*. The combination of both techniques into an assay for rapid detection of specific nucleic acid targets was already described for detection of the *mecA* gene from methicillin-resistant *Staphylococcus aureus* (MRSA) cultures [29], for direct diagnosis of *Porphyromonas gingivalis* [30], and for the identification of *Mycobacterium tuberculosis* [31].

For an optimal NALFIA result, the duplex PCR based on published results [26, 27] was slightly adjusted. In addition, FastStart *Taq* DNA polymerase was used for all experiments. Primers and polymerase concentrations were chosen to reach comparable intensities of the test lines: the *L. monocytogenes* specific line and the generic *Listeria* spp. line. By using the adapted protocol (see "Materials and methods"), the results appeared to be very reproducible. The performance of NALFIA was optimized in experiments in which the amounts of the carbon–NeutrAvidin conjugate, the concentration of immobilized capture antibody, and/or the volume of the PCR product varied.

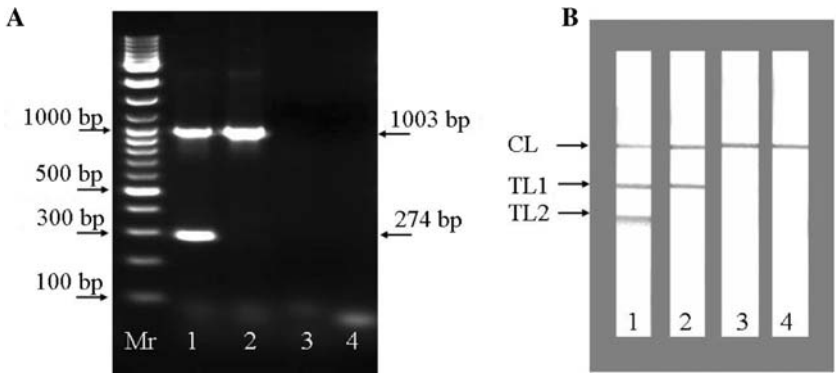

Fig. 2 Typical results of simultaneous detection of *L. monocytogenes* and generic *Listeria* spp. amplicons by agarose gel electrophoresis (**a**) and NALFIA (**b**) after duplex PCR. For both **a** and **b**, chromosomal DNA of *L. monocytogenes* (*lane 1*), *L. innocua* (*lane 2*), and *Enterobacter cloacae* (*lane 3*) was used for duplex PCR. The negative control (*lane 4*) is a primer control (PCR without template DNA). *Mr* DNA size marker, *TL1* test line specific to all species from genus *Listeria* (line of α-FL), *TL2* test line specific to *L. monocytogenes* (line of α-DIG), *CL* control line (IgG-biotin)

The specificity and sensitivity of the developed NALFIA

The specificity of the described NALFIA was studied by testing a range of *Listeria* strains and other food-relevant microorganisms (Table 1). PCR products of nine tested *L. monocytogenes* strains bound with both capture lines (the anti-DIG and the anti-FL line) and control line (CL); PCR products of all (5) other non-pathogenic *Listeria* only bound to the anti-FL line and control line. PCR products from 14 food-related, non-listerial microorganisms, the primers control (PCR without template DNA), and the buffer control (running buffer without PCR material) were negative and bound only to the control line (data not shown).

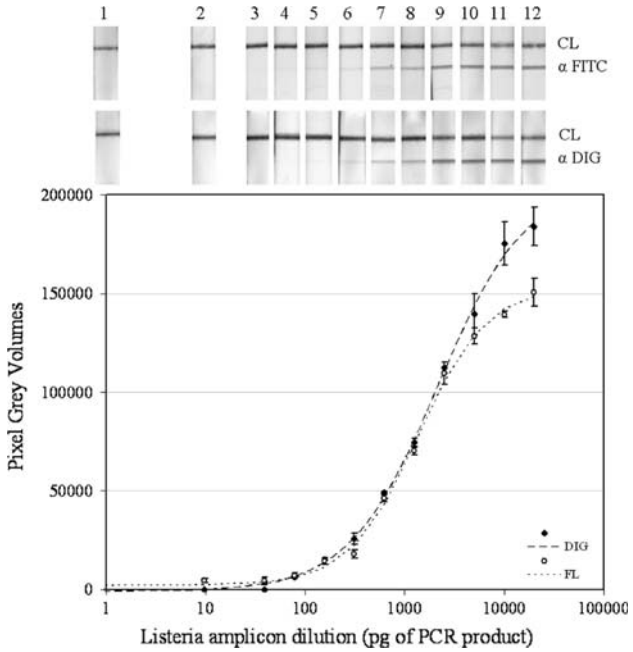

Fig. 3 Limit of detection of *Listeria*–NALFIA. Dilution series of amplified material of *L. monocytogenes* were tested by NALFIA. For generic *Listeria* detection, primers C, D (FL-labelled) were used in PCR, and for specific detection of *L. monocytogenes*, primers LIP1 and LIP2 were used (DIG-labelled). The *upper panel* shows scanned results obtained by NALFIA. The dilution range included 0 ng (*lane 1*), 0.01 ng (*lane 2*), 0.04 ng (*lane 3*), 0.08 ng (*lane 4*), 0.156 ng (*lane 5*), 0.313 ng (*lane 6*), 0.625 ng (*lane 7*), 1.25 ng (*lane 8*), 2.5 ng (*lane 9*), 5 ng (*lane 10*), 10 ng (*lane 11*), and 20 ng (*lane 12*). The *lower panel* shows the calculated pixel grey volumes after flatbed scanning and image analysis. Each point represents the mean of 3 values ± standard deviation. Results achieved with PCR material amplified with primers C, D (FL-labelled) is marked with *open symbol*, and PCR material amplified with primers LIP1 and LIP2 (DIG-labelled) with *closed symbol*

The sensitivity of NALFIA was set as the minimal concentrations of PCR amplicons that resulted in specific lines on the test membrane. Results are presented in Fig. 3 and show the dynamic range of NALFIA being 2.5–3 orders of magnitude for the DIG and FL assays. The lowest visually detectable amount was 0.1 ng of labelled amplicon.

With the optimized procedure, the lowest detectable amount of template DNA used for PCR was 50 pg of *L. monocytogenes* template DNA (Fig. 4), correlating with approximately 10^5 cells (Fig. 5), assuming a DNA content of 10^{-6} ng per cell [32]. These results corresponded to data received from experiments without pre-cultivation in which we were able to detect 10^5 cells in 1 ml of sample (Fig. 5).

Comparative costs and safety

The comparative costs and safety of electrophoresis and the typical NALFIA results are shown in Table 2. Electrophoresis is slightly cheaper in comparison with NALFIA, mainly due to the costs of labelled primers and components of immunochromatographic test (antibodies). On the other hand, NALFIA is user friendly, more rapid, less laborious (costs saving in personnel), and without any biological hazard.

Application of NALFIA to model milk samples

The *Listeria*–NALFIA technique was adapted to a tube format (see Fig. 1, [28]) to enable easy handling whilst

Table 2 Comparative costs and safety of NALFIA and electrophoresis including preparation of the sample

	NALFIA	Electrophoresis
Assessed price per 100 tests (in €)		
Cultivation, DNA isolation, PCR (without primers)	250	250
Primers	4 (labelled)	<1
Visualization	13 (antibodies, carbon)	5 (ethidium bromide)
Test components	4 (membranes, pads)	8 (agarose/gels)
Total price per 100 tests (in €)	271	264
Time for visualization of results	10 min	30–60 min
Required laboratory equipment	None	Electrophoresis station, UV transluminator, Photosystem
Labour costs (electrophoresis = 100%)	80%	100%
Biological hazard	None	Ethidium bromide

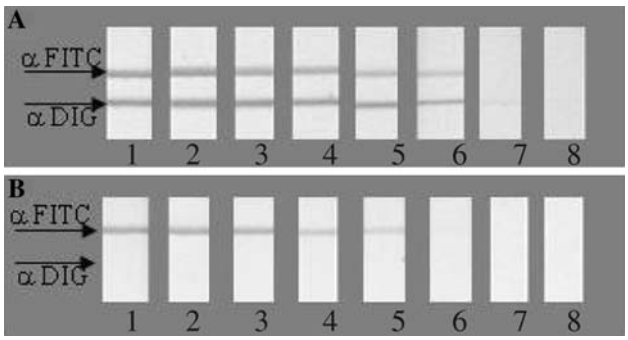

Fig. 4 Detection limit of duplex PCR-NALFIA. The detection limit of the assay was evaluated by using a diluted DNA of *L. monocytogenes* (**a**) and *L. innocua* (**b**). Dilution range included 10 ng (*lane 1*), 5 ng (*lane 2*), 2.5 ng (*lane 3*), 0.5 ng (*lane 4*), 0.25 ng (*lane 5*), 0.05 ng (*lane 6*), 0.01 ng (*lane 7*), and 0.005 ng (*lane 8*). Amplified DNA products were detected by NALFIA

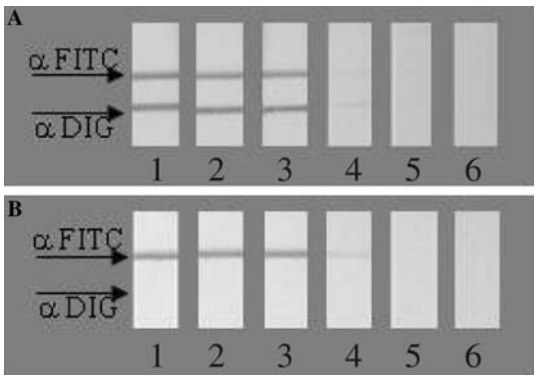

Fig. 5 Detection of cells dilutions. Multi-analyte NALFIA detection of PCR material that was prepared with DNA isolated from a dilution series of overnight cultures *L. monocytogenes* (**a**) and *L. innocua* (**b**). *1* 10^8 cells, *2* 10^7 cells, *3* 10^6 cells, *4* 10^5 cells, *5* 10^4, and *6* 10^3 cells used for isolation

reducing the overall costs of the assay. The method was finally verified by testing samples of sterilized milk that had been artificially contaminated with low amounts of different bacteria, including four different strains of *L. monocytogenes*, two strains of non-pathogenic *Listeria*, and *E. faecalis*. With the developed *Listeria*–NALFIA, less than 10 cells of *L. monocytogenes*/*Listeria* spp. were detected in 25 ml of milk within 28 h (24 h for cultivation, 4 h for PCR and NALFIA). No non-specific signals were visible when control samples (*E. faecalis* in milk, clear milk) were run (Fig. 6). The commercially available tests for specific detection of *L. monocytogenes* allow detecting *L. monocytogenes* within 37–54 h, whereas the current reference method for the detection of *L. monocytogenes* as proposed by ISO [12] allows the recovery of this pathogen in 5–7 days. The developed NALFIA procedure considerably reduces the total analysis time since the results can be obtained within 28 h from sample receipt. The application of NALFIA after cultural enrichment guarantees that target DNA is obtained from viable cells; damaged cells cannot result in false-positive lines.

Application of NALFIA to real food-related samples

The verified NALFIA was applied to a total of 24 real samples prepared from different types of food or food-related samples. The results are summarized in Table 3. Of the 24 tested samples, 15 contained *Listeria* spp. and 5 were positive for *L. monocytogenes*. All samples were also tested by the microbiological standard method and identical results as with NALFIA were obtained. Our results indicate that NALFIA can be a helpful tool for easier incorporation of molecular-biological methods as a routine diagnostic procedure for the identification of food-borne pathogens.

Conclusion

A rapid, specific, and user-friendly test for detection of *L. monocytogenes* is urgently needed by the food industry. In

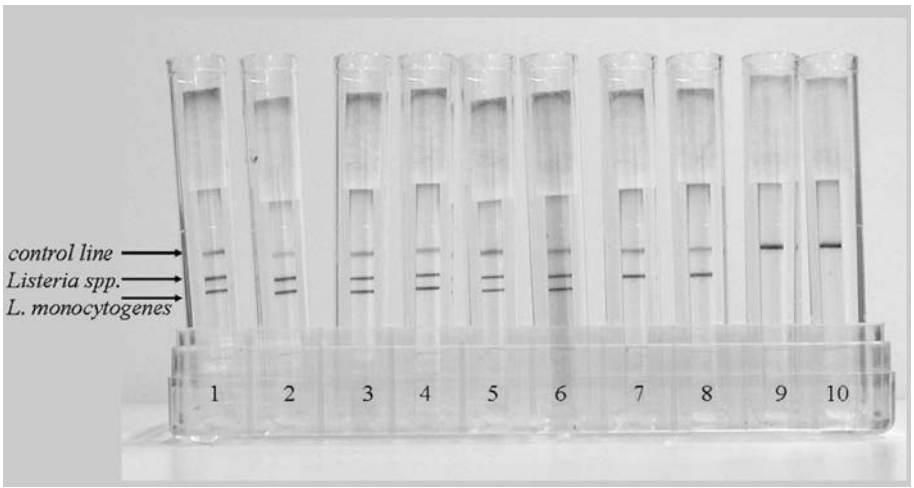

Fig. 6 *Listeria*-NALFIA in tube format. PCR material was derived from milk samples that were incurred with various bacterial (sub)species: *1 L. monocytogenes* (ALM 92, nr. 1 of triplicate), *2 L. monocytogenes* (ALM 92, nr.2 of triplicate), *3 L. monocytogenes* (ALM 92, nr.3 of triplicate), *4 L. monocytogenes* (NCTC 4886), *5 L. monocytogenes* (NCTC 4885), *6 L. monocytogenes* (milk from IFR), *7 L. innocua*, *8 L. ivanovii*, *9 Enterococcus faecalis*, and *10* negative milk control

Table 3 Results of the detection tests for listeria presence in food and biofarm samples

Food	Results	
	NALFIA	Microbiological method
Diary products		
Milk	neg	neg
Ice cream	neg	neg
Soft-dried cream cheese	Lm	Lm
Vegetable		
Broccoli	Lm	Lm
Spinach	gL	gL
Ready-to-eat salad		
Potato salad	gL	gL
Delicacy salad	neg	neg
Sprouted seeds		
Snail-clover/alfalfa	neg	neg
Mongoose	neg	neg
Nutrition for children		
Vegetables with turkey	neg	neg
Bilberries	neg	neg
Frozen bilberries	Lm	Lm
Biofarm samples		
Raw milk	gL	gL
Water	neg	neg
Silage	gL	gL
Hay	gL	gL
Faeces	gL	gL
Surface sample—nipple	neg	neg
Surface sample—floor	gL	gL
Inter-lab trials		
Pasta A	gL	gL
Pasta B	Lm	Lm
Dried milk A	Lm	Lm
Dried milk B	gL	gL
Dried milk C	gL	gL

neg negative result, *Lm* presence of *L. monocytogenes* detected, *gL* presence of bacteria from genus *Listeria* detected

a procedure in which the *Listeria*–NALFIA was applied, we have shown that the approach of using the combination of nucleic acid amplification and an immunochemical-based detection principle offers favourable advantages in terms of sensitivity, specificity, costs, and especially speed. Furthermore, since the *Listeria*–NALFIA simultaneously detects *Listeria* spp. and *L. monocytogenes*, this method will help to improve the microbiological standards of foodstuffs. This method not only helps to point at the hygienically errors in food production indicated by the *Listeria* spp. presence but it also reveals dangerous contamination by the human pathogen *L. monocytogenes*.

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