Nucleic Acid Lateral Flow Immunoassay for the Detection of Pathogenic Bacteria from Food

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Abstract: Nucleic acid lateral flow immunoassay (NALFIA) is a method combining molecular biological principle of detection with immunochemical principle of visualisation. Following isolation of DNA from the sample, a duplex PCR with two primer sets, of which one was labelled with biotin and the other with digoxigenin or fluorescein, respectively, was performed. The PCR solution and carbon particles conjugated with avidin are directly added to the nitrocellulose membrane with two test lines of immobilised antibodies specific for digoxigenin and fluorescein. The appearance of a black line indicates the presence of specific amplicon. We would like to present the NALFIA for the simultaneous detection of *L. monocytogenes* in particular and the genus *Listeria* in general, in food. Bacteria from the genus *Listeria* frequently contaminate a large variety of foods. 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 responsible for the serious illness listeriosis. Conventional microbiological methods for *L. monocytogenes* detection are laborious and take several days to achieve a confirmed identification.

Keywords: *L. monocytogenes*; Nucleic acid lateral flow immunoassay; DNA

INTRODUCTION

Lateral flow tests based on the principles of immunochromatography are currently mostly used for qualitative analysis of different types of analytes (Posthuma-Trumpie *et al*. 2008). Many variations of immunochromatographic tests are possible, but they all have in common the formation of a complex between a detector reagent bound to coloured particles that co-migrate in the sample stream and a capture reagent (e.g. antibodies) that is bound to the membrane at the position of the test line.

A new promising application of lateral flow tests is the detection of genetic material. The method is called Nucleic acid lateral flow immunoassay (NALFIA; Amerongen & Koets 2005) and is designed for testing the presence of an amplified double-stranded nucleic acid sequence specific to the analysed organism using primers with two different tags. In this case of lateral flow test, the sandwich format is applicable. Recognition of the analyte is done by binding to a tag-specific antibody (Figure 1) sprayed previously on nitrocellulose membrane and another tag-specific antibody conjugated to coloured particles enabling the visualisation.

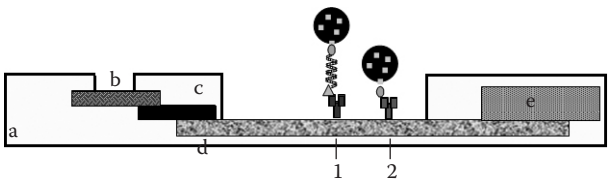

Figure 1. Schematic picture of immunochromatographic test fof nucleic acid detection: (a) plastic box, (b) sample pad, (c) conjugate pad, (d) nitrocelullose membrane, (e) absorption pad. (1) test line, (2) control line, (3) PCR product, (4) conjugate of coloured particles with streptavidine

Table 1. Primers used for duplex PCR for specific amplification of *Listeria monocytogenes* and *Listeria* spp. genetic material

Primer	Sequences	Tag	Specificity	Reference
LIP1	5'-GAT ACA GAA ACA TCG GTT GGC-3'	5' biotin-TEG 568	*L. monocytogenes*	(D'Agostino *et al.* 2004)
LIP2	5'-GTG TAA TCT TGA TGC CAT CAG G-3'	5' digoxigenin 651.8	*L. monocytogenes*	
C	5'-AGG TTG ACC CTA CCG ACT TC-3'	5' biotin-TEG 568	genus *Listeria*	(Herman *et al.* 1995)
D	5'-CAA GGA TAA GAG TAA CTG C-3'	5' fluorescein 537.5	genus *Listeria*	

MATERIALS AND METHODS

Samples preparation. Bacterial cells were cultivated overnight at 37°C and the genomic DNA extraction was performed by a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, Inc.). The isolated DNA was used as a template for a duplex PCR (MgCl 2.5mM, dNTP 0.15mM, primer LIP1/LIP2 0.1µM, primer C/D 0.2µM, polymerase 2 U, DNA 2 µl, final volume 25 µl; 25 cycles: 94°C for 30 s, 55°C for 30 s, 74°C for 1 min) with labelled primers (Table 1). Obtained PCR product, containing labelled amplicons, was run in NALFIA.

Nucleic acid lateral flow immunoassay (NALFIA). The NALFIA comprised a nitrocellulose membrane with immobilised (using LINOMAT V) polyclonal antibodies against the digoxigenin- or fluorescein-tags and colloidal carbon nanoparticles with neutravidin immobilised onto the surface. Double-labelled amplicons dilluted in running buffer (100mM borate buffer, pH 8.8, 1% (w/v) BSA, 0.05% (v/v) Tween 20, 0.02% (w/v) NaN_3) were sandwiched between the immobilised antibodies and the carbon-neutravidin conjugate.

RESULTS

The nucleic acid lateral flow immunoassay (NALFIA) for the simultaneous detection of the pathogenic *L. monocytogenes* in particular and the genus *Listeria* in general is using the combination

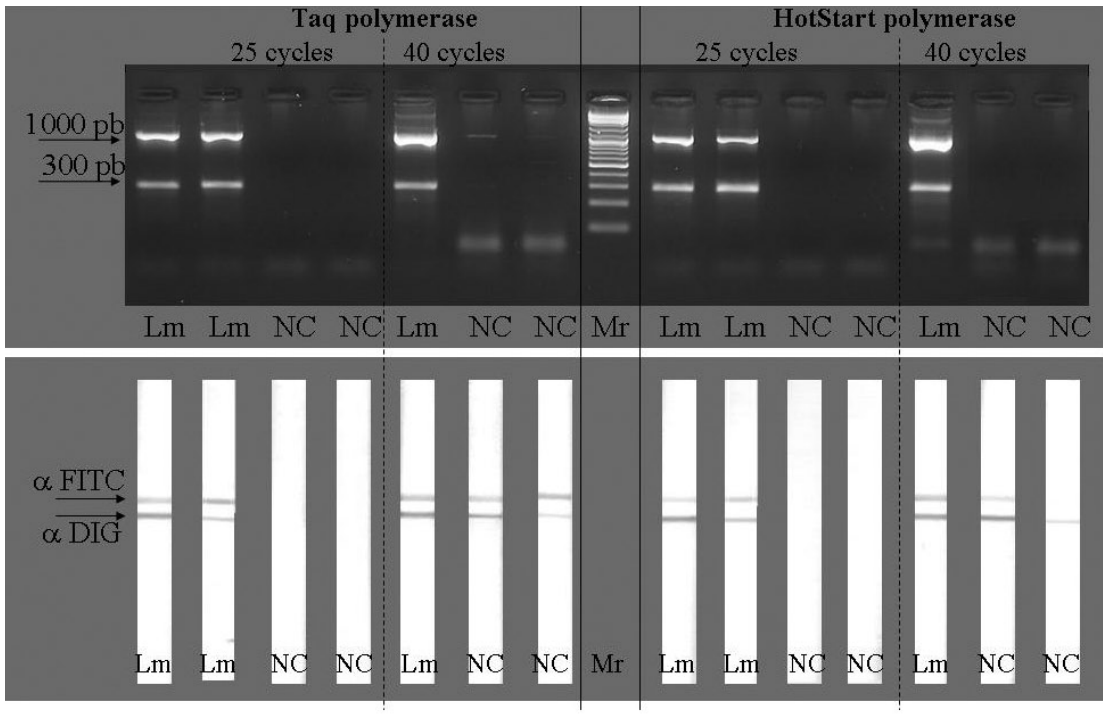

Figure 2. Influence of the number of PCR cycles and type of polymerase on the interpretation of electrophoresis (Panel A, 2% agarose gel) and NALFIA (Panel B). PCR was performed with two types of polymerase (Taq polymerase – left side, HotStart polymerase – right side). Number of PCR cycles was 25 or 40. Mr – a marker Gene ruler, Lm – DNA isolated from *Listeria monocytogenes* was used for PCR, NC – negative control (the primer control, PCR without template DNA)

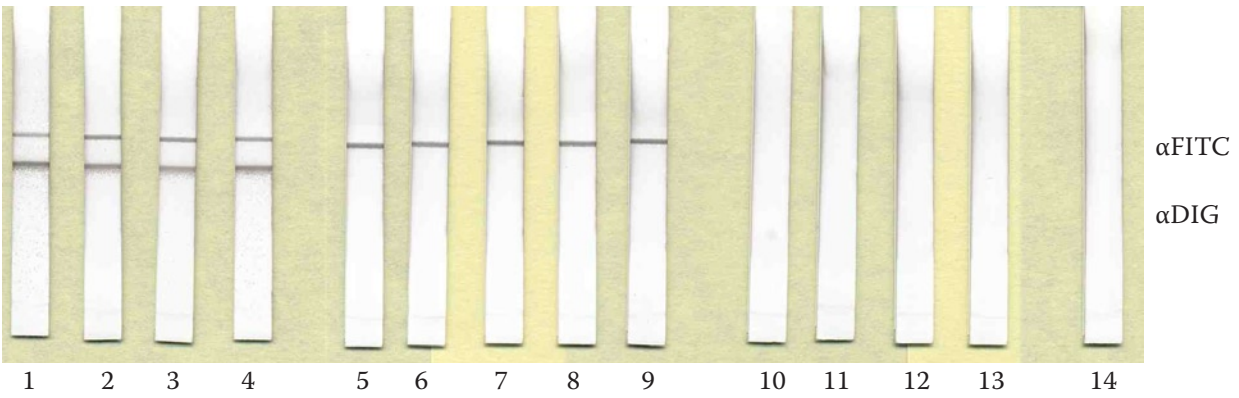

Figure 3. Specificity of *Listeria*-NALFIA

1 – *L. monocytogenes* (NCTC 4886), 2 – *L. monocytogenes* (NCTC 4885), 3 – *L. monocytogenes* (milk from IFR), 4 – *L. innocua*, 5 – *L. ivanovii*, 6 – *L. welshimeri*, 7 – *L. seeligeri*, 8 – *L. grayi*, 9 – *Bacillus cereus*, 10 – *Enterobacter cloacae*, 11 – *Enterococcus faecalis*, 12 – *Lactobacillus plantarum*, 13 – PCR no template control

of nucleic acid amplification and an immunochemical based detection principle. The detection procedure starts with enrichment of the sample. Following isolation of DNA a duplex PCR is performed with two labelled primer sets. 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 the specific amplicon. Samples containing the *L. monocytogenes* specific double-labelled amplicons were indicated by the appearance of two grey/black lines; samples containing the *Listeria* spp. specific double-labelled amplicons were indicated by the appearance of one grey/black line.

Duplex PCR for simultaneous amplification of both generic *Listeria* spp. and specific *L. monocytogenes* sequences was based on Herman *et al.* (1995) and D'Agostino *et al.* (2004). Although these PCR protocols were suitable for the detection of amplicons on agarose gels (Figure 2), false positive results were initially observed in the *Listeria*-NALFIA. This problem was eliminated by decreasing the amount of PCR cycles from 40 to 25 cycles and by using Fast Start Taq DNA polymerase instead of the conventional Taq DNA polymerase. FastStart Taq DNA polymerase is a thermostable, modified form of recombinant Taq DNA polymerase, with which the occurrence of nonspecific amplification products can be substantially reduced.

The specificity of the described NALFIA was studied by testing a range of *Listeria* strains and other food relevant microorganisms in artificially contaminated milk samples. PCR products of all tested *L. monocytogenes* strains bound with both capture lines (the αDIG and the αFITC line). PCR products of all other nonpathogenic *Listeria* only bound the α-FITC line. PCR products from other microorganisms (*Bacillus, Enterococcus, Enterobacter*, and *Lactobacillus*), and the primer control (PCR without template DNA) were all negative (Figure 3).

CONCLUSION

The lateral flow tests are becoming more and more popular for testing of wide range of analytes. The lateral flow tests offers many benefits (user-friendly format, short time to get test result, long-term stability, and relatively low price). A new promising application is the detection of genetic material, e.g. various genetic markers, DNA or RNA specific for infectious disease pathogens.

The aim of this short note was to present a new method NALFIA that can be used for rapid detection of pathogenic microorganisms (presented *Listeria monocytogenes*) in food. The NALFIA can be used as a modern elegant tool for easy and clear interpretation of molecular biological tests. The horizontal electrophoresis usually used for evaluation of this kind of tests employs very often dangerous chemicals (ethidium bromide) and the result is dependant on the analyst who makes the measurement. The NALFIA enables to prevent both, furthermore the time of performace is much shorter for the NALFIA comparable to eletrophoresis.

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