Talanta 101 (2012) 405–412

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Detection of food-borne pathogens with DNA arrays on disk

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ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form
10 September 2012
Accepted 22 September 2012
Available online 28 September 2012

Keywords:
DNA array
Discs
Food-borne pathogens
Optical detection

ABSTRACT

A DNA oligonucleotide array for duplex pathogen detection on a DVD platform is developed. The assay involves hybridization of PCR products and optical detection using compact disc technology. Different DNA array constructions for attachment of synthetic oligonucleotides on to DVD surface are evaluated, finding that streptavidin–biotin coupling method yielded the highest sensitivity in combination with enzymatic signal amplification. Issues of importance for the DNA array construction such immobilized probes design, PCR product labeling strategy and composition of the hybridization buffer were addressed. The methodology was proved scoring single nucleotide polymorphisms with high selectivity. The assay capability was also demonstrated by the identification of two pathogenic microorganisms in powder milk samples. In fifty minutes, the DVD-array system identifies Salmonella spp. and Cronobacter spp. (previously named Enterobacter sakazakii) precise and simultaneously with a sensitivity of 10^0 and 10^2 cfu/mL, respectively, in infant milk. Results were in good agreement with those obtained by quantitative real-time PCR.

1. Introduction

Accurate and quick detection and quantification of pathogen microorganisms in food, drinking water and in clinical diagnostics using cost-effective methodologies are greatest challenges currently facing food industry, health and environmental fields. The World Health Organization (WHO) deals with the need of controlling and preventing pathogen toxic infections associated to food consumption. In this sense, public awareness of food safety issues and the continued focus of the European Food Safety Authority (EFSA) on EU preventing systems, make the development of rapid and low cost sensing tests of great interest. Indeed, the European Union with a population of more than 500 million is a significant market for food microbiology testing. According to a recent report [1], an estimated 275 million food micro tests were conducted only in Europe in 2011 and is expected to reach close to 350 million tests in 2016.

Salmonella spp. and *Cronobacter* spp. are categorized by the WHO as potentially dangerous microorganism contaminants. Intrinsic contamination of milk powder with these microorganisms is an important cause of serious illness in infants. In recent years, several outbreaks of *Salmonella* infections in infants have been linked to the consumption of powder infant formula, resulting in diarrhea, bacteremia and meningitis [2]. Also, *Cronobacter* spp. is considered to be an emerging human pathogen of life-threatening bacterial systemic infection in neonates [3,4]. In this context, milk powder has been suggested in several reports to be the vehicle responsible for 50–80% of *Cronobacter* spp. infections [5].

Numerous technologies have been developed to detect and identify specific pathogens in foods [6–8]. The current gold standard method of detection and quantification of pathogens involve culturing the microorganisms in selective media and identifying isolates according to their morphological, biochemical and immunological characteristics. This conventional method remains lengthy and labor intensive, disadvantages in many industrial applications, particularly in the food sector.

Immunological based techniques have been successfully employed for the detection of food-borne pathogens [9]. In spite of their short assay time compared to the traditional one, antibody–antigen based methods show disadvantages mainly related with the difficulty to raise specific antibodies for the detection of microorganisms at the species level and the high cost of monoclonal antibody production [10]. In contrast, nucleic acid-based methods offer several advantages over the traditional as specificity and sensitivity for rapid on-site detection of food-borne pathogens [11]. Among them, PCR-based amplification methods are widely employed for pathogen detection [12] once the appropriate primers are designed. In spite of their advantages, from an industrial point of view routine detection of pathogens using PCR can be expensive and complicated, requiring full equipped laboratories. Also, at the moment there is a limitation

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http://dx.doi.org/10.1016/j.talanta.2012.09.049

in the number of targets that can be detected in a single reaction without compromising the sensitivity.

Alternatively, DNA oligonucleotide arrays coupled with PCR methods have been introduced and developed as potential strategies for facilitating high-throughput and specific screening of pathogen-associated DNA sequences [13–15]. These methods have the advantage of detecting simultaneously thousands of DNA probes in a single reaction and identify several microorganisms with good sensitivity, selectivity and high throughput capacity. However, the high cost of the detection benchtop equipments is the main drawback, making unfeasible for routine testing. The food industry requires sensitive, low cost and rapid methods in order to control the safety of their products. To this aim, easy to handle and cheap techniques that provide results in a quick way at a competitive value and enable the analysis of a large number of samples are demanded.

In this context, a consolidated alternative based on microarray technology is the use of compact discs that shows great potential, especially in combination with a disc drive as detector [16]. This strategy is very suitable for designing affinity-based arrays of high density given the advantages such as (a) huge analytical area where thousands of probes can be deposited and dozens of samples analyzed simultaneously [17], (b) mass manufacturing of high quality materials at a very low price (approximately, ten cents of euro per disc), (c) possibility of integrating both numerical and microbiological information at the same analytical platform where the analysis is developed and (d) use of an standard optical drive as chemical detector that is commercialized at very low price, highlighting its ubiquity, robustness, ease of use, portability, working capacity and in situ operation. In this sense, several analytical applications using DVD technology for the determination of environmental contaminants, antibiotics, allergens, etc., have been reported in the last 3 years [18].

The aim of this work was to develop a rapid and sensitive duplex DNA array on a DVD platform for the simultaneous detection of *Salmonella* spp. and *Cronobacter* spp. in powder infant milk after PCR amplification as a double checking sensor system. To our knowledge is the first approach using DVD technology for duplex detection and identification of food-borne pathogens. For that, different strategies for DNA microarray construction are evaluated, reaching good sensitivity. Also, probe design, PCR product labeling strategy and the choice of hybridization conditions are crucial points to be optimized before DNA array construction for precise and sensitive detection of microorganism contaminants.

2. Materials and methods

2.1. Bioreagents

DNA concentration and quality were determined by measuring the optical density at 260/280 nm with a NanoDrop ND 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware). Real-time PCR was performed with the Bio Rad iCycler iQTM Multicolor Real Time PCR Detection System (Bio Rad Laboratories, Hercules, CA). DNA primers were acquired from TIB MOLBIOL (Berlin, Germany). The PCR mixture contained PCR amplification buffer, $MgCl_2$, Taq Platinum DNA polymerase (Invitrogen, Carlsbad, CA), dNTPs (Applied Biosystems, Foster City, CA), SYBR Green I (Invitrogen Life Technologies, Carlsbad, CA) and DIG-11-UTP (Roche Diagnostics, Mannheim, Germany).

The PCR amplification products were analyzed by the Agilent 2100 BioanalyzerDNA assay (Agilent Technologies, Palo Alto, CA), a microfluidic platform that automatically sizes and quantifies PCR fragments accurately and reproducibly. Streptavidin, horseradish peroxidase (HRP) and gold labeled streptavidin were purchased from Sigma (Madrid, Spain). HRP labeled anti-digoxigenin antibody was from Abcam and the gold labeled from Aurion (Wageningen, Holand).

2.2. DNA attachment on disc

Two strategies were used to immobilize DNA probes on DVDs. A scheme of the strategies is shown in Fig. 1. One employs specific antibodies to attach 5′digoxigenin modified probes (SAL3; hybridization assay HA1) and the other streptavidin to link 5′ biotin modified oligos (SAL1; hybridization assay HA2). In all cases, the bioreceptors were immobilized by physisorption on the DVD surface. For that, bioreceptor/probe solution in 1:2 M ratio was prepared in spotting buffer (0.1 M sodium bicarbonate/carbonate buffer, pH 9.0).

Before spotting, the DVD discs (MPO Ibérica, Madrid, Spain) were first conditioned by gentle ethanol washing, water rinsing and dried by spinning for 1 min at 800 r.p.m. Spotting was performed using a liquid dispenser robot (Biodot AD1500, Irvine, CA), depositing 25 nL of bioreceptor/probe solution. The size of the spots was ~500 μM in diameter and 1.0 mm apart. The spotted disks were incubated 16 h at 4 °C and then washed with PBS-T (10 mM sodium phosphate buffer 0.15 M NaCl, 0.05% Tween 20, pH 7.4), rinsed with MilliQ water, and dried.

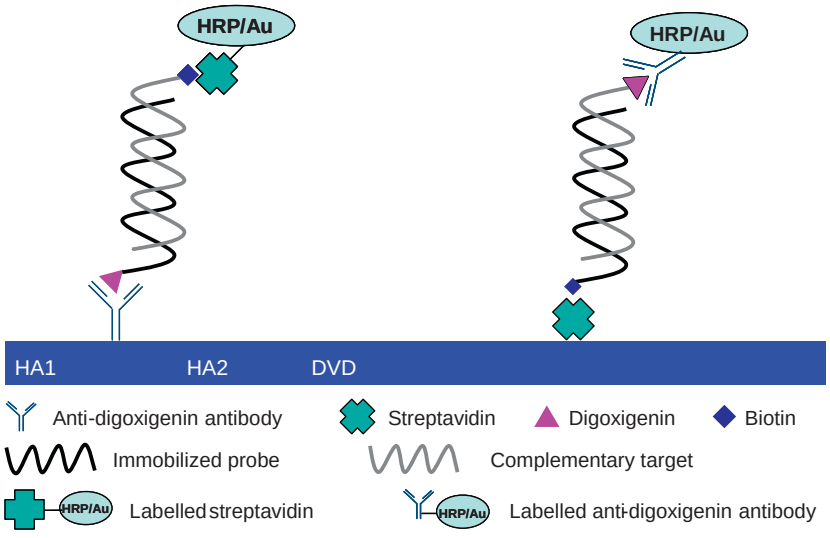

Fig. 1. Scheme of hybridization assays.

Disks were segmented in ten areas each one containing nine arrays of 3×3 spots. In this way, ten different samples can be analyzed simultaneously.

2.3. DNA amplification

For the *Salmonella* spp. detection the primer pair consisting of LHNS 531 (5′–TACCAAAGCTAAACGCGCAGCT-3′) and RHNS 682 (5′–TGATCAGGAAATCTTCCAGTTGC-3′) was used to amplify a 152 bp region of the Salmonella *hns* gene as previously described [19]. Primers design was done with PRIMER3, v0.3.0 and IDT SciTools PrimerQuestSM (Integrated DNA Technologies, Inc.).

The real-time PCR assay was performed on a real-time thermocycler iQ5, Bio Rad, Hercules, CA. The selectivity of the primers and probes was tested on a total of eight *Salmonella* strains (*S. enterica* serotype Typhimurium CECT 443, *S. enterica* serotype Typhimurium grup B –CCUG 21390S-, *S. enterica* serotype Lille CCUG 126472, *S. enterica* serotype Dublin CECT 415, 2 strains of *S. enterica* serotype Enteritidis and 2 strains of *S. enterica* serotype Typhimurium isolated from clinical samples) and twelve non *Salmonella* species (*Campylobacter jejuni* CCUG 17696, *Citrobacter freundii* CECT 401, *Enterococcus faecalis* CECT 4081, *Enterobacter aerogenes* CECT 684T, *Escherichia coli* ATCC 10536, *Hafnia alvei* CECT 157, *Klebsiella pneumoniae* CECT 142, *Listeria monocytogenes* CECT 4032, *Proteus vulgaris* CECT 484, *Pseudomonas aeruginosa* CECT 116, *Serratia marcescens* ATCC 274 and *Staphylococcus aureus* CECT 239).

The PCR mixture for both *Salmonella* spp. and *Cronobacter* spp. contained 1 u Taq polymerase, 3 mM magnesium chloride, 300 nM of primers, a deoxynucleoside triphosphate mixture (0.1 mM each of dATP, dCTP, dGTP; 0.065 mM dTTP; 0.035 mM DIG-11-dUTP) and 0.1 *x* SYBR Green I in the PCR reaction buffer. The real-time PCR program was started with a denaturation step of 7 min at 95 °C, followed by 35 cycles at 95 °C (30 s), 62 °C (30 s) and 72 °C (30 s) and a final cycle at 72 °C for 4 min.

For the *Cronobacter* spp. detection the primer pair consisting of SI-3F (5′–GTTGGATCACCTCCTTACCTGC-3′) and SI-Rev (5′–AGTTAAACCTCTTCAACT CCTG-3′) was used to amplify a 190 bp region of the *Cronobacter* spp. 16S ribosomal RNA gene (Gi:55501732). The selectivity of the primers and probes was tested on a total of four *Cronobacter* species (*Cronobacter sakazakii* ATCC BBA-894, *Cronobacter dublinensis* subsp. Dublinensis LMG 23823T, *Cronobacter malonaticus* LMG 23826T and *Cronobacter turicensis* LMG 23827T) and twelve negative control for *Cronobacter* genera (*Salmonella* serotype typhimurium group B –CECT 443-, *Salmonella* serotype Lile group C –CCUG 12467-, *Salmonella* serotype Dublin group D –CECT 4152-, *Salmonella* serotype Anatum group E-CCUG 36820-, *Salmonella* serotype Minesota grup L –CCUG 21390-, *Citrobacter freundii* CECT 401, *Escherichia coli* CECT 10536, *Proteus vulgaris* CECT 484, *Klebsiella pneumoniae* CECT 142, *Enterobacter aerogenes* CECT 648T, *Hafnia alvei* CECT 157, *Serratia marcescens* ATCC 25419). The real-time PCR program was started with a denaturation step of 7 min at 95 °C, followed by 35 cycles at 95 °C (20 s), 57 °C (20 s) and 72 °C (20 s) and a final cycle at 72 °C for 4 min.

The DNA that was used as a template was previously isolated from *Salmonella* spp. and *Cronobacter* spp. pure cultures. For the selectivity tests, bacteria strains were grown aerobically at 37 °C for 18 h. Viable counts were obtained by culturing each strain overnight at 37 °C on nutrient agar plates.

2.4. Hybridization protocol

Hybridization on disc was performed using complementary 5′biotin labeled oligo (SAL4) or using 5′digoxigenin labeled complementary target (SAL2) for hybridization assays HA1 and HA2, respectively. Table 1 shows the sequences of the oligonucleotides used to detect Salmonella spp. (SAL) and Cronobacter spp. (ENS).

Fifty microlitters of complementary oligo or PCR product diluted in $2 \times$ SSC ($1 \times$ SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0), containing 50% formamide (v/v), pH 7.0, were applied to each disc area and glass coverslips (18×18-mm) were used for dispersion and cover, reducing the chance of evaporation and avoiding local background. After incubation in a slim box for 30 min at 37 °C, the coverslips were gently removed and the disk washed with deionized water. The hybridization event was detected using HRP and gold labelled streptavidin or anti-digoxigenin antibody tracers for assays HA1 and HA2, respectively. For that, 1 mL of 1/1000 dilution of tracers in PBS-T was dispensed on to the disc and then a 12 cm diameter dummy plastic surface was used for solution dispersion. After 15 min incubation at room temperature, the cover surface was removed, and the disc washed with deionized water. Next, hybridization event was developed by dispensing 1.0 mL TMB (3,3′,5,5′-tetramethylbenzidine) or silver enhancement for HRP and gold labelled strategy, respectively, distributing the solution evenly along the whole disc surface as before. The reaction was stopped after 5 min by removing the dummy plastic surface and washing the disc with water. The colored insoluble product obtained modifies the optical properties of the disc allowing for the detection employing a DVD drive as chemical detector. After DNA extraction and PCR amplification, the total assay time, including hybridization, detection and scanning the disc was 50 min. 2.5. Spiked food samples preparation.

Table 1
Sequences, fusion temperature and type of oligonucleotide used to detect and identify *Salmonella* spp. and *Cronobacter* spp.

	Sequence 5′-3′	T_m (°C)	Oligonucleotide	Assay
SAL1	**Bt**$(T)_{10}$TTTgATTACAgCCggTgTACgACCCT	75.9	Probe (26 bases)	HA2
SAL2	**Dx**AgggTCgTACACCggCTgTAATCAAA	72.2	ADN target	HA2
SAL3	**Dx**$(T)_{10}$TTTgATTACAgCCggTgTACgACCCT	75.9	Probe(26 bases)	HA1
SAL4	**Bt**AgggTCgTACACCggCTgTAATCAAA	83.6	ADN target	HA1
SAL5	**Bt**AgggTCgTACA*T*CggCTgTAATCAAA	71.0	Probe(26 bases)	HA2(SNP)
SAL6	**Bt**AgggTCg*C*gCAC*TAT*CTgTAATCAAA	70.7	Probe(26 bases)	HA2(SNP)
SAL7	**Bt***ACC*gTCg*C*gCAC*TAT*CTg*ATT*TCAAA	73.7	Probe(26 bases)	HA2(SNP)
SAL8	**Bt**$(T)_{10}$TTCCATTgCTTTTTTgATTACAgCCggTgTACgACCCT	81.5	Probe(38 bases)	HA2
SAL9	**Bt**$(T)_{10}$CTTACCTTgTTCTTCCATTgCTTTTTTgATTACAgCCggTgTACgACCCT	83.6	Probe(50 bases)	HA2
SAL10	**Bt**$(T)_{10}$ATCTTCCAgTTgCTTACCTTgTTCTTCCATTgCTTTTTTgATTACAgCCggTgTACgACCCT	85.7	Probe(62 bases)	HA2
SAL11	**Bt**$(T)_{10}$AgggTCgTACACCggCTgTAATCAAA	75.0	Probe(26 bases)	HA2
ENS1	TgTgAgCACgCgAggTTgTATCTTgCA$(T)_{10}$**Bt**	73.2	Probe (27 bases)	HA2
ENS2	TgCAAgATACAACCTCgCgTgCTCACA**Dx**	69.4	ADN target	HA2

Bt: Biotin, Dx: Digoxigenin. The sequences with bases in italics and underlined are the mismatches.

The presence of *Salmonella* spp. and *Cronobacter* spp. in powder skimmed milk samples was determined by using DVD technology and quantitative real-time PCR (qRT-PCR) as reference method. For spiked food samples preparation, 10 mL of powder milk, purchased at local market, were separately inoculated with 10-fold serial dilutions of *Salmonella* spp. and C*ronobacter* spp. reference strains, to obtain from 10^4 to 10^0 cfu/mL of milk. The powder milk samples were previously reconstituted and assayed to confirm the absence of wild *Salmonella* spp. and/or *Cronobacter* spp. The pre enrichment step was avoided and genomic DNA was straight isolated from 1 mL of the spiked milk samples.

2.5. DNA extraction

For the DNA extraction 1 mL of milk was centrifuged at 300 g for 3 min to remove insoluble milk components. The supernatant was centrifuged again at 20.000 g to pellet the microorganims. The supernatant was then carefully removed and the pellet is resuspended in the buffer supplied by the extraction kit, DNeasy Blood & Tissue (Qiagen GmbH, Hilden, Germany). The rest of the protocol steps were performed according to the manufacturer's instructions for the bacterial DNA isolation. The final centrifugation was done in 50 μL of the DNA elution buffer instead of the 100 μL recommended in the kit instructions.

2.6. Disc scanning and data analysis

The DVD drive used in this study was from LG Electronics Inc. (Englewood Cliffs, NJ), which was controlled by custom software (Diskpick) running on a personal computer, and connected to it through a USB2.0 universal serial bus interface. Briefly, during the DVD reading, the laser hits the hybridization product which modifies the reflection properties of the DVD surface, attenuating laser beam intensity that reaches the photodiode of the pickup [16]. The attenuated analog signals are directly acquired from the photodiode of the DVD drive, being the optical density of the reaction product related to pathogen presence.

To scan the surface of the DVD (5 min at 16x speed), the software simulates the writing process of a 4.4 GB size file. During the disk scanning, only signals coming from selected areas are processed for digitization, stored in the computer (2 MB size file) and deconvoluted into an image. Diskpick software was written in Visual C++ to control the optical disk drive, (sampling rate, detector gain, spatial resolution, and scanning speed) and identifies spots with S/N ≥ 3 (limit of detection) to then calculate the mean signal intensity by averaging data points from a circular area of 50 μm in diameter. Moreover, this software allows for exporting the results. For sample analysis, the measured signal intensity was related to the presence of pathogen in the sample.

3. Results

The construction of DNA based microarrays on optical discs for bacterial sensing requires a systematic optimization of several parameters to obtain sensitive and selective assays. First, the study started by evaluating the capability of attaching specific probes indirectly onto the disc. Two strategies were approached both based on the adsorption of bioreceptors (streptavidin and antibodies) to further attach 5′modified probes for sensing *Salmonella* spp. as a model system. The immobilization efficiency of probes for both strategies was evaluated by hybridization assays, using complementary targets (50 pM–10 μM) that were labeled either with digoxigenin (SAL2) or biotin (SAL4) depending on the approached strategy. The concentration of oligo probes (SAL1 and SAL3) varied from 5 nM to 250 nM and the concentration of bioreceptors (STV and anti-digoxigenin antibody) from 15 nM to 300 nM. As negative controls STV and probe solutions were separately spotted in the disc. Hybridization assays HA1 and HA2 reached a sensitivity of 0.8 and 2.2 μM, respectively, using silver amplification. The results obtained using enzymatic amplification were promising, reaching a limit of detection (S/N = 3) of 100 nM and 350 pM, respectively. These values were obtained from the calibration curve using complementary targets. In terms of sensitivity, the results indicated that in a regular basis the enzymatic detection was better than the silver amplification using gold labeled tracers. Also, strategy HA2, employing 100 nM/200 nM streptavidin/probe spotting solution was much more sensitive strategy than HA1, dealing with the detection of full match complementary target. This molar protein/probe ratio gave the best results in terms of sensitivity for all hybridization assays tested caused probably to immobilization density.

The immobilization density of probes was estimated indirectly calculating the amount of protein immobilized on disc by using ATTO655 fluorescent labeled streptavidin. For that, serial dilutions of the labeled protein (0.01–10 nM) were spotted on disc and the fluorescent readouts were immediately taken using a CCD camera. The fluorescent intensity of the spots was quantified using the arrayscan software to obtain a standard curve for the protein. An estimated percentage of immobilized protein on disc was extracted from the calibration curve after washing the disc that was previously printed with labeled protein (25–250 nM) and incubated for 16 h at 4 °C. The calculations rendered a protein immobilization on disc of 0.2%. Taking into account that initial probe/streptavidin molar ratio was 2 and the protein is the limiting reagent, an estimated immobilization probe density of 0.05 f mol/mm^2 was calculated. This value is in good agreement with that obtained using fluorescent labeled probe immobilized by streptavidin–biotin coupling. In comparison to other anchoring strategies, based on covalent attachment of probes on discs, the immobilization density is similar and suitable to perform hybridization assays [20]. The reason why other probe/streptavidin ratios did not improve assays sensitivity might be due to the fact that probe attachment depends directly on protein immobilization efficiency.

3.1. DNA amplification

The results of all the Salmonella strains PCR tests were positive, while the non-Salmonella strains that were tested yielded negative results, with neither a Cq nor a DNA product, as expected. The same results were obtained for all the Cronobacter species tested, DNA amplification results were positive, while the other non-Cronobacter spp. gave negative results. Therefore, the two PCR reactions demonstrated to be highly selective for each pathogen.

3.2. Analysis of mismatches probes

The selectivity of the assay for *Salmonella* spp. was tested by studying the ability to discriminate synthetic oligonucleotide probes. The assay consisted in immobilizing a mix of streptavidin (100 nM) and DNA probes (200 nM) with 1 (SAL5), 5 (SAL6) and 10 (SAL7) (Table 1) mismaches. The oligonucleotide SAL-1 (200 nM) was arrayed as a negative control. In order to study the hybridization performance, an additional probe (SAL11 at 200 nmM) containing 10 thymines as spacer arm was also included in the array.

The discrimination capability was evaluated by comparing the detection signals obtained from the mismatched probes with that of the full match probe (SAL4 at 200 nM) after hybridization with target (SAL3 at 500 nM). The hybridization assay, detection and amplification were developed as described above. For that, the

effect of the composition of hybridization buffer with regard to formamide percentage (10, 25 and 50%) was also studied since is critical to selectively discriminate probes with only one mismatch [21]. Probes SAL6 and SAL7 were fully discriminated with 10% formamide in the hybridization buffer giving S/N below 3, while probes SAL4 and SAL11 gave 35 and 52, respectively. The discrimination of SAL5 (1 mismatch) required a more selective media that included 50% formamide in the hybridization assay. Under this condition, probe SAL5 was fully discriminated with S/N below 2, being 33 and 50 for SAL4 and SAL11, respectively. This result shows that specific DNA detection is possible by the regulation of formamide percentage in the hybridization buffer, discriminating DNA probes with only one mismatch. It is also worth mentioning that the assay reaches a higher signal-to-noise ratio when using immobilized probes containing a spacer arm compared to that without spacer arm (SAL4), rendering a slightly more sensitive assay. This fact indicates that part of the probe is hindered as a consequence of STV coupling, partially limiting the recognition event to its complementary target.

3.3. Optimization of recognition event conditions

A systematic study to evaluate the effect of the different parameters involved in the hybridization process was also performed using *Salmonella* spp. as a model pathogen. Concentration of monovalent cation (Na^+), formamide percentage, temperature and incubation time were the variables studied. In terms of sensitivity and selectivity, hybridization in 2 × SSC, pH 7.0, with 50% formamide at 37 °C during 30 min rendered the best results. The presence of formamide in the hybridization buffer was necessary to reduce the stability of dsDNA since the affinity between the two complementary strands is influenced by this additive. Also, formamide made the assay more selective (see SNP section) without losing analytical signal. Thus, 50% of formamide was the optimum additive concentration since lower percentages made the assay less selective. On the other hand, lower temperatures made less specific assays and temperatures above 42 °C limited the sensitivity of the assays.

Regarding hybridization time, periods larger than 30 min did not improve S/N ratios. The reason might be due to the low sample volume used that generates a thin liquid film over the sensing area of 100 μM and because of DNA diffusion rate allowing for reaching the equilibrium at short incubation times.

The applicability of the sensor system on disc was evaluated sensing PCR products. A key aspect to take into account in a hybridization assay with PCR products is the way how double strand DNA is denaturalized. For that, three denaturation protocols stated as physical (A), chemical (B) and particles (C) were compared in terms of sensitivity, using SAL1 immobilized probe. The denaturation protocol A consisted in heating the PCR product during 5 min at 95 °C before hybridization on disc. The chemical dsDNA dissociation was based on the use of sodium hydroxide (0.3 M) during 5 min and further pH adjustment with 1 M HCl. The third denaturation prorotocol used commercially available magnetic particles with streptavidin immobilized on them. This denaturation protocol is only valid to PCR products containing biotin. For this evaluation, five types of PCR products were prepared using DNA template extracted from *Salmonella* spp. plate culture under the PCR protocol conditions described in Materials and Methods.

A scheme of the different PCR products is shown in Fig. 2A. PCR2–5 products were tested using the hybridization assay HA2 and PCR1 by HA1. PCR1 resulted from the amplification of DNA template using 5′biotin labeled forward primer; PCR2 was obtained after amplification of DNA template using 5′digoxigenin labeled forward primer; PCR3 as a result of amplification of DNA template using 5′biotin labeled forward primer and digoxigenin labeled dUTPs; PCR4 after amplification of DNA template using 5′digoxigenin labeled forward primer with digoxigenin labeled dUTPs, and PCR5 product was obtained after amplification of DNA template using non-labeled primers and digoxigenin labeled dUTPs.

In Table 2 the sensitivities of the hybridization assays using the all PCR products are shown. It can be concluded that magnetic particles strategy is good and comparable to regular denaturation protocols (see results of PCR3) in terms of sensitivity. However, the hybridization assays were not satisfactory when using the physical and chemical denaturation protocols, probably due to the lower sensitivity of HA1 in comparison to HA2. Also, the dsDNA separation using this method is laborious and time consuming in comparison to chemical and physical protocols. The best results were obtained with the physical protocol in combination with the use of PCR4 and PCR5, reaching a sensitivity of 60 and 50 μg/L, respectively. The most probable reason of these findings is the number of digoxigenin molecules per mole of PCR product when comparing the results obtained with the hybridization assay using PCR2 that incorporates one molecule of digoxigenin.

The length of the probe is a critical variable in the sensing response not only for the specificity of the hybridization and the stability of the double strand, but also for the kinetics of the reaction on disc. The hybridization rate is mainly determined by the access of the targets to the immobilized probe, which is influenced by its density, the diffusion rate and the length of the probe. The later variable is determinant to obtain a sensitive assay that at the same time states sample volume and the number of amplification cycles. For that, the effect of the immobilized probe length on hybridization signal was studied by attaching biotin modified 26 (SAL1), 38 (SAL8), 50 (SAL9) and 62 (SAL10) mer probes. First, hybridization magnitude was determined by using a full match 26 mer complementary target. In this case, similar results were obtained in terms of sensitivity for all immobilized probes. This result indicates that 62 mer probes immobilized on disc maintain similar capability to specifically recognize target as the shortest probe (SAL1) does. More interesting results were obtained when detecting PCR5 products. In this case, hybridization of a PCR product of 152 bp size specific for *Salmonella* spp. was the goal. In this study, the hypothesis of using longer probes was to move away the 3′ tail of the PCR product from the sensing surface, reducing possible steric hindrances and improving hybridization signal as the number of complementary bases implied in the recognition event probe-amplicon increased. A schematic representation of the effect of probe length in targeting the complementary strand of PCR products is shown in Fig. 2B.

Under the optimum conditions, PCR5 products obtained after amplification of DNA template extracted from Salmonella spp. isolates (10^{10} cfu/mL) were serially diluted (1/250, 1/500 and 1/1000) in hybridization buffer (2 × SSC, 50% formamide, pH 7.0) and analyzed on disc as described in Materials and Methods section. As is shown in Fig. 2C, the length of the immobilized probe was critical to detect the PCR product at low concentration, showing that signal intensities increase with the length of the immobilized probe. Thus, 1/1000 dilution, corresponding to 15 μg DNA/L, gave a S/N of 31 (SAL10), whereas sensing with probe SAL1 the assay reached a S/N of 20 for 60 μg DNA/L (1/250 dilution), indicating that the length of the immobilized probes is determinant for increasing hybridization assay sensitivity. The S/N sensing PCR5 product with SAL9 and SAL10 probes are similar and in all cases above the limit of quantification of the sensor system ($S/N \geq 10$).

At this point the sensitivity of the assay to detect PCR products was determined by analyzing DNA extracted from sets of genomic DNA prepared as templates from 10 fold serial dilutions that

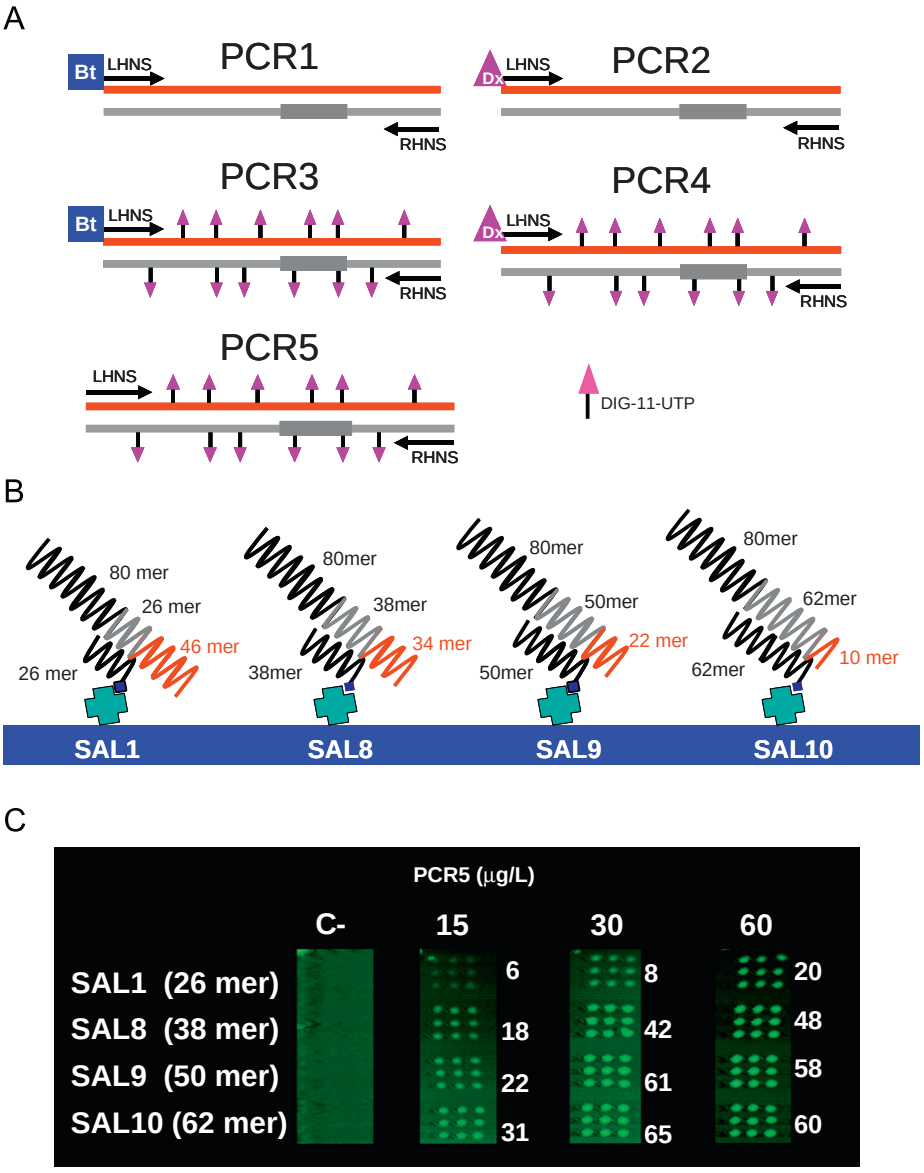

Fig. 2. (A) Illustration of the types of PCR products. The thicker gray fragment at the DNA template corresponds to the immobilized probe. (B) Schematic representation of the effect of probe length in the hybridization of the complementary sense strand of the PCR product. The PCR fragment in gray corresponds to the complementary sequence. As the length of the immobilized probe increases, the number of complementary nucleotides of the PCR product grows in 3′direction, in order to move away from the disc surface (fragment in red). (C) Image obtained after scanning the disc. The panel C^- corresponds to the image obtained with the negative control. The number at the right of each array corresponds to the obtained signal-to-noise ratio. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Limit of quantification of the hybridization assays of PCR products.

	Denaturation protocol		
PCR product	Physical	Chemical	Magnetic particles
PCR1	–	–	200*
PCR2	90	150	n.a
PCR3	110	130	140
PCR4	60	90	n.a
PCR5	50	90	n.a

* Values are expressed in µg DNA/L. n.a: not applicable.

ranged from 10^4 GE (Genomic Equivalents) to 10^{-1} GE of a pure Salmonella Typhimuriun culture using SAL10 as immobilized probe. The sensitivity of the DVD array was related to the signal-to-noise ratio (S/N) calculated from background signal. The hybridization assay on disc detected DNA extracted from 10^{-1} cfu/mL with S/N of 41, which was 15.7 times the limit of detection of the assay, allowing for the detection of pathogen at very low levels. Next, in order to quantify the analytical performances of the sensor system serial dilution of a PCR product with a concentration of 4.0 µg/L was performed and analyzed by hybridization assay. The dilutions tested were 1/125, 1/500, 1/2000, 1/4000, corresponding to 32, 8, 2 and 1.0 µg/L of DNA and the signal-to-noise ratios obtained were 70, 29, 16 and 11, respectively. Assay reproducibility was also determined from the optical density of spots. To determine intradisk and disk-to-disk relative standard deviations (RSDs) the signals obtained after the analysis of PCR products (1/125 dilution), corresponding to 10^2 and 10^0 cfu/mL were analyzed. For that, four DVD arrays were tested. The intradisk RSD varied from 10.1 to 11.4%, whereas the disk-to-disk RSD ranged from 12.5 to 13.7%. These results

corroborate the good performances of the DVD array to detect genomic DNA at μg/L level, reaching a limit of quantification ($S/N=10$) of 1.0 μg/L, corresponding to 19.8 pM.

3.4. Determination of pathogens in powder milk samples

A two-laboratory validation study was performed by analyzing powder skimmed milk samples. The methods were based on DVD technology and quantitative real-time PCR (qRT-PCR). For the analysis using DVD array methodology, the samples were separately spiked with Salmonella spp. and Cronobacter spp. isolates and after DNA extraction and amplification PCR5 type products were obtained. For hybridization, 1 μL of each PCR product was diluted in 100 μL of hybridization buffer (2 × SSC, 50% formamide, pH 7.0) and analyzed in duplicate in the disc. The DVD layout is shown in Fig. 3. No amplification was observed in negative controls, which indicates no false positive results. Absence of PCR inhibitors was demonstrated by the amplification of the positive control. As it can be seen in Table 3, the comparison results show that the DNA oligonucleotide array on disc detected 10^0 cfu/mL of Salmonella spp. and 10^2 cfu/mL of Cronobacter spp.. The difference in sensitivity observed in disc for the simultaneously detection of both food-borne pathogens might be due to the shorter immobilized probe length used for the identification of Cronobacter spp. The design of longer immobilized probes for this pathogen was unsatisfactory, obtaining negative hybridization results probably due to the formation of hairpins or stem-loop structures within the probe.

4. Conclusions

The present study is the first work to demonstrate the use of compact disk technology in combination with nucleic acids amplification for the detection and identification of food-borne pathogens in food samples as a sensitive, rapid, low cost and portable sensing system. Duplex DNA oligonucleotide array developed on a DVD platform is a suitable screening tool, offering several advantages as a flexible and ease-to-use method for rapid on-site detection of food-borne pathogens. The sensitivity, specificity and reproducibility of the assays support also the use of this methodology in routine analysis in the food industry.

Streptavidin–biotin coupling method onto DVD in combination with enzymatic signal amplification allows for a general and systematic methodology to determine other dangerous microorganism contaminants at one time. Probe design for array construction is critical and limits the sensitivity of the assay. Results of optimization experiments demonstrated that 50–62 mer oligonucleotide probes on the DVD array were highly sensitive and selective. The amplifier labeling during PCR is also a determinant parameter in order to achieve good results. Thus, the use of labeled dNTPs during the PCR is the better choice for pathogen sensing on a DVD. The signal intensities corresponding to each pathogen in milk samples were at least 10-fold higher than the background levels. Also, the DVD array is a good analytical platform capable of discriminating probes with only one mismatch, indicating the selectivity of the assay under stringent conditions.

The DNA oligonucleotide DVD-array is especially suitable for screening applications with low investment equipments, maintaining the analytical performances obtained with more sophisticated methodologies such as quantitative real-time PCR. Also, the proposed compact disk based PCR-DVD array methodology may suit well with isothermal amplification methodologies to facilitate implementation in a point of care setting. Finally, we anticipate that compact disk laboratory equipment capable of

Table 3
Duplex determination of food-borne pathogens.

c.f.u/mL	Salmonella spp.		Cronobacter spp.	
	qRT-PCR	DVD	qRT-PCR	DVD
0	−	−	−	−
10^0	+	+	−	−
10^1	+	+	−	−
10^2	+	+	+	+
10^3	+	+	+	+
10^4	+	+	+	+
positive control	+	+	+	+
negative control	−	−	−	−

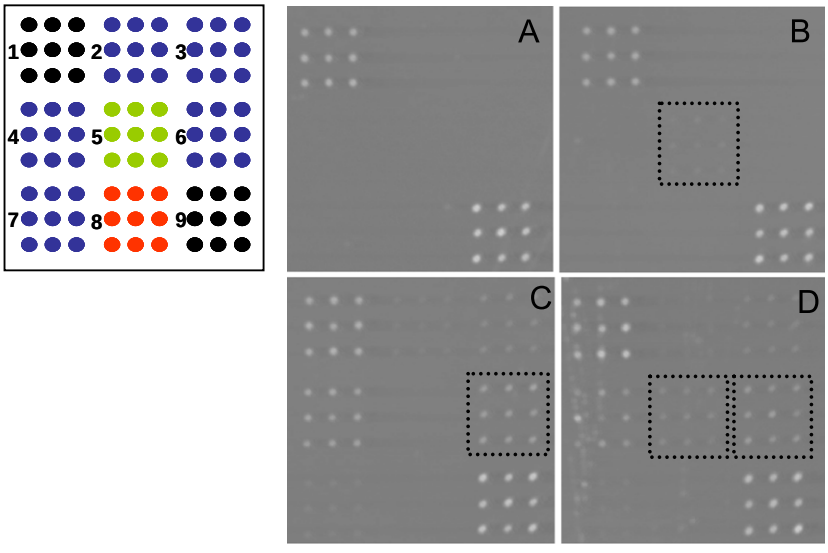

Fig. 3. Duplex determination of food-borne pathogens. Probes immobilized on to the DVD platform correspond to positive control (1), SAL1 (2), SAL8 (3), SAL9 (4), ENS1 (5), SAL10 (6), SAL11 (7), negative control (8) and positive control (9). SAL11 is the specific probe for the detection of the anti-sense strand of the Salmonella spp. amplifier. Panels A, B, C and D correspond to control, 10^2 c.f.u./mL of *Cronobacter* spp., 10^0 c.f.u./mL of *Salmonella* spp., and duplex assay at the above microorganisms concentration. The subarrays are framed for better visualization of the results.

DNA amplification and visual colorimetric detection will be a valuable tool also in other fields such as clinical diagnostics and biothreat detection.

Acknowledgments

This work was funded by the projects PATSENS, PSE-010000-2008-6 (Spanish Government and EU FEDER funds), FEDER CTQ2010-15943 (CICYT, Spain), PROMETEO 2010/008 and ACOMP/2012/158 (Generalitat Valenciana). The Spanish MEC provided T.A-C with a grant for her PhD studies.

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