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Detection of food-borne pathogens with DNA arrays on disk

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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.

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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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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.

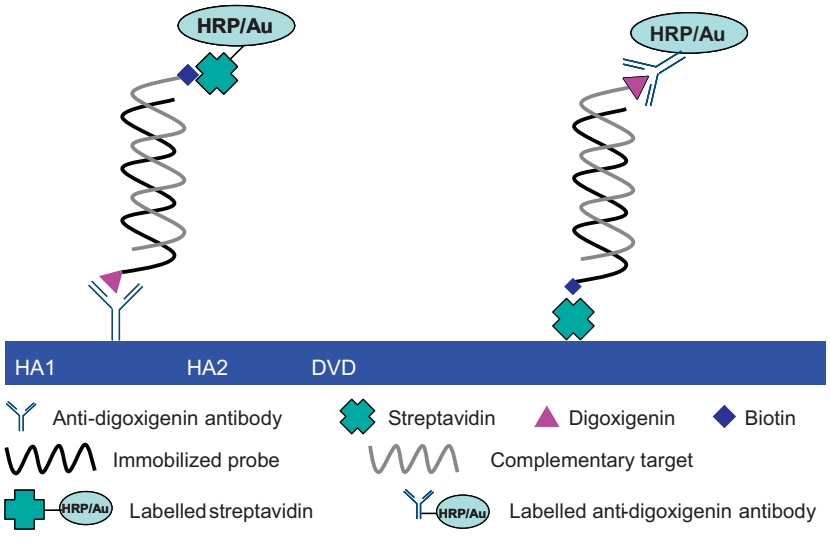

Fig. 1. Scheme of hybridization assays.

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