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Isothermal DNA amplification strategies for duplex microorganism detection

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ABSTRACT

A valid solution for micro-analytical systems is the selection of a compatible amplification reaction with a simple, highly-integrated efficient design that allows the detection of multiple genomic targets. Two approaches under isothermal conditions are presented: recombinase polymerase amplification (RPA) and multiple displacement amplification (MDA). Both methods were applied to a duplex assay specific for *Salmonella* spp. and *Cronobacter* spp., with excellent amplification yields (0.2-8.6 10^8 fold). The proposed approaches were successfully compared to conventional PCR and tested for the milk sample analysis as a microarray format on compact disc (support and driver). Satisfactory results were obtained in terms of resistance to inhibition, selectivity, sensitivity (10^1-10^2 CFU/mL) and reproducibility (below 12.5%). The studied methods are efficient and cost-effective, with a high potential to automate microorganisms detection by integrated analytical systems working at a constant low temperature.

Keywords: isothermal DNA amplification; pathogens; milk; microarraying

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1. Introduction

The development of effective detection methods for pathogenic microorganisms covers several areas, such as food safety, environmental monitoring and clinical diagnostics. Microbiological methods are replaced with those based on DNA, where amplification with polymerase chain reaction (PCR) is the most widespread approach. For instance, automation of DNA amplification enables the use of portable microdevices, multiplexing, reduced sample volumes and reagents, and prevents contamination risks (Asiello, & Baeumner, 2011). Efforts are being made to overcome its drawbacks for extended point-of-need applications; for example, increasing multiplexing ability or reducing costs, time analysis and technical requirements. Yet the integration of nucleic acid amplification into microdevices, such as digital PCR or lab-on-a-chip, is complex, and several issues must be resolved. PCR demands accurate temperature control and rapid thermocycling at between 55ºC and 95ºC. When initiating a specific step in the PCR, temperature fluctuation results in over- and under-shooting (Kim, Yang, Bae, & Park, 2008). High temperatures also lead to variations in the volume reaction and gas bubble formation, which are the main causes of PCR failure in lab-on-a-chip devices (Nakayama, Hiep, Furui, Yonezawa, Saito, Takamura, & Tamiya, 2010). Consequently, the design of simple, cost-efficient systems is no trivial matter, particularly when integrating sample preparation and/or multiplex detection into the same platform.

The use of enzymes mimicking DNA replication *in vivo* conditions is an alternative to conventional DNA polymerases (Gill, & Ghaemi, 2008). Thus amplification can be performed using a simple thermoblock, peltier or oven at a fixed temperature. The commonest isothermal methods are strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), helicase-dependent amplification (HDA), isothermal recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP), and multiple displacement amplification (MDA) (Zanoli, & Spoto, 2013, Yan, Zhou, Zheng, Gamson, Roembke, Nakayama, & Sintim, 2014). The performance of RPA (Piepenburg, Williams, Stemple, & Armes, 2006) and MDA (Dean et al., 2002) offers a quite interesting high-throughput analytical system, as demonstrated in a bright approach (digital RPA) proposed for the detection of a single pathogen on a chip (Shen, Davydova, Du, Kreutz, Piepenburg, & Ismagilov, 2011). However, these methods have not been described for multiplex strategies.

Several factors should be considered when integrating an amplification reaction for a high-capacity analytical platform, such as microarrays, including the compatibility of

the amplification mechanism with multiplexing detection because other isothermal reactions are intrinsically limited to one target analyte (e.g., LAMP). Very few data describing analytical performances are available given the novelty of these amplification methods for analytical purposes. For instance, information about properties, such as temperature tolerance or the effect of inhibitors from a sample matrix, is scarce or even null.

The present research work deals with the potential integration of RPA (sequence-specific method) and MDA (massive method) into a duplex system. This study, for which *Salmonella* spp. and *Cronobacter* spp. were the chosen targets, was based on food safety and environmental monitoring or clinical diagnostics (Cahill, Wachsmuth, Costarrica, & Embarek, 2008, Hyeon, Park, Choi, Holt, & Seo, 2010, Derzelle, & Dilasser, 2006, Wang et al., 2009). As there is clear evidence for a causal association between the presence of *Salmonella* spp. and *Cronobacter* spp. in food and illness in humans, infections from these microorganisms have been documented as both sporadic cases and outbreaks (Codex Alimentarius Commission, 2008). Several methods have been proposed to determine the presence/absence of these specific bacterial pathogens. Contamination of infant milk has been extensively reported, based on traditional microbiological examination and DNA-based techniques, including culture enrichment and PCR amplification. The method was applied in milk samples to evaluate duplex isothermal amplification. Furthermore, DVD (Digital Versatile Disc) has been used as low-cost, integrated effective microarray platforms and detection technology (Morais, Tortajada-Genaro, Arnandis-Chover, Puchades, & Maquieira, 2009, Siegrist, Peytavi, Bergeron, & Madou, 2010) to demonstrate the concept by simultaneously quantifying both pathogens with a view to future screening applications (allergens, GMOs, species identification, etc.).

2. Material and methods

2.1. Amplification protocols

The target gene *hns,* which codes for a DNA-binding protein, was selected because it is conserved in all the *Salmonella* spp. The specific target for the *Cronobacter* spp. species was located in the 16S-23S rDNA internal transcribed spacer sequence.

PCR mixtures (25 µL) consisted of 5 ng of extracted genomic DNA, 1× Tris-KCl buffer (100 mmol/L Tris-HCl, 500 mmol/L KCl, pH 8.3), 2 mmol/L $MgCl_2$, 200 µM dNTPs, 1.25 units of Taq DNA polymerase (Roche, Mannheim, Germany) and 400 nmol/L of

each primer (Table S-1). The thermal program was: denaturation (95ºC, 7 min) followed by 40 cycles of denaturation (95ºC, 30 s), annealing (59ºC, 30 s) and elongation (72ºC, 30 s), and a final elongation (72ºC, 4 min).

RPA reactions (25 µL) were performed by adding 5 ng of genomic DNA from inoculated milk samples and 240 nmol/L of the primer pairs (Table S-1) to the reconstituted solution of enzymes, nucleotides and buffer (TwistDx, Cambridge, UK). The duplex reactions were carried out in an oven (40ºC, 40 min).

MDA reactions (25 µL) were performed with final concentrations of 2 ng of genomic DNA, 1× MagniPhi buffer reaction (X-Pol Biotech, Madrid, Spain) and 50 µmol/L of random hexamer primer. After heating (95ºC, 3 min), 500 µmol/L dNTPs, plus phi29 polymerase (1 U), were added and the reaction was run in an oven (35 ºC, 4.5 h) until a final inactivation step at 65ºC (10 min).

2.2. Bacterial strains, milk samples and DNA extraction

Salmonella serovar Typhimurium group B (CECT 443) and *Cronobacter sakazakii* (ATCC BBA-894) were used as reference strains (positive controls). Milk products, bought in local food stores, were inoculated with both pathogens. Inoculation assays were prepared by adding 10-fold serial dilutions of an 18-hour culture in sterile saline solution (0.8% NaCl) to cover a range from 0 to $4 \cdot 10^4$ CFU g^{-1} for each pathogen. Genomic DNA was extracted from bacterial cultures and samples using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA, USA).

2.3. Analysis of amplification products

Amplification products were separated by electrophoresis on 3% (w/v) agarose gel, 1× TBE buffer (89 mmol/L Tris base, 89 mmol/L borate, 2 mmol/L EDTA, pH 8) at 120 V and room temperature. Gels were stained for 30 min with 1× TBE containing 0.01% (v/v) of SYBR-Safe (Life Technologies, Carlsband, CA), and bands were visualized on an UV transilluminator. Size was determined by comparing with a 50-bp ladder. Single amplification yields were calculated from the fluorescence measurements with SYBR-Safe at 0.01% (v/v) in a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Turku, Finland).

2.4. Post-amplification protocol

Two post-amplification protocols were assayed: restriction enzyme digestion and sonication.

The EcoNI enzyme (Fermentas, Vilnius, Lithuania), also called XagI, produced the double-stranded cut of sequence CCTNN-N-NNAGG, which was closed to the target region of both pathogens. The distance to the target region was 41 bp for *Salmonella* spp. and 92 bp for *Cronobacter* spp. The digestion conditions were optimized: temperature (30-45 ºC), time (1-16 h) and restriction enzyme units (1-4 U). The optimal protocol (37 ºC, 8 h and 2 U of restriction enzyme) was performed in a total volume of 32 µL by adding 3.2 µL of 10× digestion buffer (10 mmol/L Tris-HCl, 10 mmol/L MgCl2, 100 mmol/L KCl, 0.1 mg/mL BSA, pH 8.5), 10 µL of amplification product and 3.2 µL of the EcoNI enzyme solution (20 U). After the reaction, the restriction enzyme was inactivated by incubation at 65ºC for 20 min.

For the second option, small DNA fragments were obtained by sonication (UP200S ultrasonic disruptor, Hielscher, Teltow, Germany) using a microtip (1 mm in diameter) by applying 10 cycles (30 s ON, 30 s OFF) at 24 the kHz operating frequency, pulse 0.5 s and 70% amplitude. Vials were cooled in an ice bath to maintain sample integrity.

2.5. Inhibition assays

The amplification yield was evaluated in the presence of potential inhibitors. To that end, skimmed cow's milk samples and powdered infant formulas were added to the amplification mixtures to obtain a final concentration ranging from 0.05% to 20% (v/v). The effect of the Ca^{2+} ion was determined by adding $CaCl_2$ (0.2-8 mmol/L) to the amplification solutions.

2.6. Addressing a biosensor based on DVD technology

Amplification products were analyzed by DVD-technology (Tortajada-Genaro, Santiago-Felipe, Morais, Gabaldón, Puchades, & Maquieira, 2012). Streptavidin (10 mg/L) and 5'-biotinylated probes (50 nmol/L), listed in Table S.1, were spotted on the polycarbonate surface of a digital versatile disk (DVD), and 10 arrays were printed (6×6). Amplified products (1 µL) were mixed with 49 µL of hybridization buffer (NaCl 750 mmol/L, sodium citrate 75 mmol/L, formamide 25%, pH 7), heated (95ºC, 5 min) and dispensed onto sensing arrays to perform the simultaneous analysis of 10 samples. After hybridisation (37ºC, 60 min) and washing, 1 mL of anti-digoxigenin antibody-HRP solution in PBS-T (1: 500) was dispensed onto the DVD (room temperature, 30

164 min). After washing, 1 mL of the 3,3',5,5'-tetramethylbenzidine solution was dispensed
165 and incubated for 8 min. After the recognition process and the developer reaction, the
166 disc was placed into the DVD-drive and scanned by laser, and reflected light was
167 measured. In the absence of a solid deposit (reaction product), the reflection properties
168 of the DVD surface remained unchanged and the maximum intensity of the reflected
169 beam was collected by the DVD drive (background signal). However when the laser hit
170 a microarray spot, the reflected laser beam attenuated and, consequently, the laser beam
171 intensity that reached the photodiode of the DVD pickup diminished (Figure S-1). By
172 means of data acquisition software, a microarray image was generated, and the signal of
173 each spot correlated with pathogen concentration. Assay sensitivity was established by
174 analysing the bacterial DNA extracts obtained by serial dilution (0.1 to 10^5 CFU/mL).
175 Limits of detection (LODs) were calculated as the pathogen concentration that produced
176 a signal-to-noise ratio of 3. Assay reproducibility, expressed as relative standard
177 deviations (RSDs), was calculated from triplicates. The microarray layout (6×6) on a
178 compact disc consisted in four blocks (specific for *Salmonella* spp., specific for
179 *Cronobacter* spp., positive control and negative control) of nine dots each. With this
180 arrangement, the 50-nL printing solution yielded spots of 500-µm diameter and a track
181 pitch (centre to centre distance) of 1.5 mm. An array density of about 1.0 spot mm^{-2} was
182 achieved (Figure S-2).
183
184 **2.7. Quality control**
185 Firstly, a parallel analysis of control samples was performed (Arnandis-Chover, Morais,
186 Tortajada-Genaro, Puchades, Maquieira, Berganza, & Olabarria, 2012). Blank cultures
187 and non-inoculated milk samples were included as negative controls. A mixture with the
188 genomic DNA from the cultures of both pathogens (*Salmonella* spp. and *Cronobacter*
189 spp.) was used as the positive sample. The results show the right amplification,
190 hybridisation and detection protocol (external control). Secondly an oligonucleotide,
191 non-complementary to the target pathogens, (negative block) and a digoxigenin-labelled
192 oligonucleotide (positive block) were added in the array layout. These internal controls
193 informed whether the detection protocol in the same array that hybridizes the DNA
194 from the unknown sample was successful.
195 The duplex amplifications performed with the specific primers of the two target
196 pathogens were compared to the single amplifications containing the specific primers

for one pathogen (specific *Salmonella* spp. or specific *Cronobacter* spp.). No performance differences were observed between both study approaches.

3. Results and discussion

3.1. Amplification methods

In the RPA reaction, multi-variable initial experiments for the duplex assay (*Salmonella* spp. and *Cronobacter* spp.) revealed that primer concentration, incubation temperature and reaction time were the most critical parameters. Figure 1A shows that the individual concentration of the primers in a duplex assay can lower if compared to those used in the single assay, probably because the maximum reaction rate was achieved. Regarding kinetic behaviour, the amplification process reached a stationary phase after 40 min (Figure 1B). A similar amplification yield was obtained when the oven was working within the 37-42ºC range, showing high tolerance to temperature fluctuations. Therefore, the selected concentrations of primers were 240 nmol/L and the largest number of copies was reached at 40±2ºC for 40 min, which corresponds to an amplification yield of $8.6 \cdot 10^8$. No false-positive results were obtained due to pre-initiation or non-specific amplification.

Furthermore, RPA is a sequence-specific amplification method that requires the design of primers, similarly to PCR, to control product properties or selectivity. The initial experiments showed that the distance between the forward and reverse primers had a major effect on amplification yield. Therefore, product length varied, and the forward primer specific to *Salmonella* changed, while the rest of the reaction conditions remained constant. Figure 1C shows the number of copies which lowered with product length, but the reaction rate increased. These results were interpreted according to the RPA mechanism (Piepenburg, et al. 2006) and polymerase processivity; i.e., measurement of the global number of nucleotides added per time unit (Zhuang, 2010). A short product involves cutting the time that proteins are bound to the DNA compared to the proteins that are free in the solution, and the total number of nucleotides incorporated lowers. However, template replication finished early and, consequently, the exponential amplification was favoured, and the number of copies for the target sequences increased. It is worth mentioning that this effect is important for isothermal methods because processivity in PCR can be controlled by changing the elongation step time.

RPA specificity was checked at different levels. Firstly, an alignment against closely related species was performed by the Blastn software (NBCI databank). The forward primer of *Salmonella*, used to amplify the 100-bp product, was rejected because it aligned against other pathogens, such as *Escherichia*, *Shigella* and *Photorhabdus*. The set of specific oligonucleotides to the 152-bp product for *Salmonella* spp. and the 190-bp product for *Cronobacter* spp. were selected for the duplex amplification method. Secondly, the analysis of pure bacterial cultures, listed in Table S-2, was satisfactorily done and provided negative amplification results for the non-target pathogens.

In the MDA reaction, a non-primer design was necessary because phi29 polymerase combined with a random hexamer randomly amplified the whole genome (massive amplification method). The mechanism involved strand displacement DNA synthesis on single- and double-strand DNA templates by primer annealing at multiple sites. Therefore, the amplification yield depended mainly on primer concentration, primer nature, temperature and reaction time. Reproducible results were obtained for hexamer concentrations under 50 μmol/L, which was the optimum value (Figure 2A). The primers resistant to the exonuclease activity of phi29 polymerase (the thiophosphate linkage for two 3' terminal nucleotides) provided good efficiencies as compared to the exo-sensitive primers (non-internal modification). The results also indicated that phi29 polymerase was less tolerant to temperature fluctuations because the number of copies changed according to the working temperature (Figure 2B). Finally, the reaction time study showed that a stationary phase was reached after 270 min. Therefore, the best yield, $9.8 \cdot 10^4$ in genomic units, was reached at 35.5±0.5ºC for 4.5 h.

The results of both duplex methods were comparable to single pathogen approaches, and amplification yields were highly reproducible (variation < 5%). Low liquid evaporation and gas-bubble formation were observed at working temperatures. Thus these amplification methods are technically simpler for miniaturised systems, and are less sensitive to temperature fluctuations than PCR.

3.2. The post-amplification protocol

Duplex RPA reactions yielded the two predicted products, as confirmed by agarose gel electrophoresis. The multibranched polymerisation mechanism of MDA leads to a massive amplification method that provides several products. The bands in the electrophoretic separation appeared as smears, which were evenly distributed from 0.3 to 4 kbp for the mixtures of both pathogens. In this case, integration with microarraying

platforms was limited because the good stability of the large-sized products resulted in low hybridisation yields, as described for PCR-based methods (Halperin, Buhot, & Zhulina, 2006).

Further fragmentation of amplification products prior to the hybridisation assays was required. To that end, enzymatic and physical protocols were assayed. For the first option, restriction enzyme EcoNI was selected because a common sequence (CCTNN-N-NNAGG) was presented for both target regions (gene *hns* in *Salmonella* spp. and the 16S-23S rDNA internal transcribed spacer sequence in *Cronobacter* spp.). This enzyme provided fragments from 0.1 to 0.8 kbp after 8 h of digestion. Comparable amounts of small-sized oligonucleotides (Figure 3) were obtained by sonication (high frequency acoustic waves >20 kHz). Although sonication is a fast automatable option for MDA, the absence of a post-amplification treatment in the RPA approach simplifies its integration into a high-throughput platform.

3.3 Analytical features. Addressing a biosensor based on DVD technology

To date, the RPA method has been reported only for single determinations using end-point fluorescent detection, lateral-flow strips or microfluidic chips (Shen et al., 2011, Lutz et al., 2010). The MDA method has been used for whole genome amplification in combination with functional gene arrays (Dean et al., 2002, Erlandsson, Rosenstierne, McLoughlin, Jaing, & Fomsgaard, 2011). The reached LODs were between 10 and 1000 copies/mL. However, very few described approaches are quantitative methods and they have not been applied as real multiplex approaches, such as microarray platforms.

By way of example of a simple portable detection system, the combination of isothermal amplification with compact disc technology was firstly studied. The results indicated that this analytical platform and the detector were highly compatible with isothermal amplification approaches because no further DNA product treatment, for example purification, was required. A duplex assay to simultaneously detect *Salmonella* spp. and *Cronobacter* spp. was also easily implemented in a microarray format on a DVD surface following the protocols described in previous studies (Tortajada-Genaro et al., 2012). The positive and negative controls were included to guarantee the reliability of the pathogen analysis.

The results were compared to those obtained with the PCR-based method, and showed a good correlation between the amount of DNA and the optical signal. Thus, the LODs for *Salmonella* spp. were 17-32 copies/mL for PCR, 10-48 copies/mL for RPA and 7-31

298 copies/mL for MDA (Table 1). Although the amplification factor of MDA was lower
299 than RPA, the LODs were similar in the microarraying format, probably because of the
300 amplification mechanism. In RPA, denaturation was performed by a mixture of
301 enzymes and polymerisation generated double-strand DNA (Piepenburg et al., 2006).
302 Amplification by the MDA method involved strand displacement DNA synthesis on
303 templates (Dean et al., 2002). Then the formation of single-stranded regions during
304 multibranched polymerisation should increase the hybridisation yield. Intra-day
305 reproducibility was lower than 8.5% for PCR, and below 12.5% for RPA and MDA.
306 Inter-day reproducibility ranged from 6.3% to 16.8%. The analytical performances
307 obtained with both amplification methods, without an enrichment culture, were similar,
308 or better, than those obtained by RT-PCR, glass microarrays or traditional
309 microbiological methods.
310
311 **3.4 Milk sample analysis**
312 It is well-known that some enzymes used for amplification are not compatible with the
313 specific substances present in the sample matrix, which diminishes their activity and,
314 subsequently, assay sensitivity (Wilson, 1997). For this reason, resistance to inhibition
315 for the three studied amplification approaches was assessed in milk samples (Table 2).
316 The RPA method amplified even in presence of 15-25% of milk, while the MDA
317 method provided less tolerance to the matrix (14-16%). Moreover, all the studied
318 amplification methods showed similar inhibitions to those caused by Ca^{2+}, where 6.3-
319 7.2 mmol/L was the maximum concentration tolerated. This inhibitory effect has been
320 previously reported for PCR (Al-Soud, & Rådström, 1998), but has never been
321 described in isothermal polymerases.
322 Powdered infant formulas and skimmed milk were spiked with *Salmonella* spp. and
323 *Cronobacter* spp. (0 to $4 \cdot 10^4$ CFU/mL). Non-inoculated milks were negative, whereas
324 all the inoculated samples were positive, and a correlation between pathogen amount
325 and optical intensities was found (Figure 4). The obtained recovery levels were in good
326 agreement with the spiked concentration in all cases (Table 3). It is worth mentioning
327 that the methods did not require an overnight enrichment step, which considerably cut
328 the analysis time and allowed duplex detection in the samples with pathogens under 40
329 CFU/mL. The proposed approaches open up an advantageous form of pathogen
330 determination using automated devices. The development of a competitive, portable,
331 low-energy analytical system that integrates all the steps is underway.

4. Conclusions

RPA and MDA are two innovative methods that offer several advantages for the automation of DNA assays in wide range of point-of-need applications. Isothermal amplifications do not require sophisticated hardware for accurate temperature control against thermocycling PCR-based methods. Moreover, both enzymes operate near room temperature if compared to other isothermal reactions (e.g., LAMP at 60ºC). The results reveal that the studied amplification methods offer portability or cost-effectiveness without compromising analytical performance, tolerance to inhibitors or price per assay. Nevertheless, some properties, such as short times or lack of a post-amplification protocol, indicate RPA has a higher potential than the MDA method for point-of-need applications. MDA is also an interesting method for high-multiplexing determination because RPA, such as PCR, is limited by a restrictive primer design and to a small number of targets (<10 genes).

A duplex system using these isothermal amplifications is firstly proposed. This study also demonstrates that the integration of nucleic acid amplification and detection into analytical devices, such as compact discs (bio-recognition and reading), is technically possible and allows high-throughput analyses. Therefore, the low-cost detection of different targets in parallel and minimal manipulation comes closer to such approaches.

ASSOCIATED CONTENT

Additional figures are described in the *Supplementary Information*.

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FIGURE CAPTIONS

Fig. 1. Effect on amplification efficiency for the duplex RPA method (*Salmonella* spp. $4{\cdot}10^1$ CFU/mL and *Cronobacter* spp. $4{\cdot}10^1$ CFU/mL): (A) individual primer concentration, (B) temperature and time, and (C) amplification product length varied, while the forward primer specific to *Salmonella* changed, and the rest of working conditions remained constant.

Fig. 2. Effect on amplification efficiency for the duplex MDA method (*Salmonella* spp. $4{\cdot}10^1$ CFU/mL and *Cronobacter* spp. $4{\cdot}10^1$ CFU/mL): (A) primer concentration and (B) temperature and time.

Fig. 3. Fluorescence response of small-sized products (< 800 bp) measured after post-amplification treatment and gel electrophoresis separation.

Fig. 4. The microarray signals yielded by amplification techniques for inoculated milk samples with both pathogens: (A) Average spot density for *Salmonella* spp. and (B) Average spot density for *Cronobacter* spp.

TABLE CAPTIONS

Table 1. Comparison of the experimental protocols and analytical performances obtained by the PCR, RPA, and MDA techniques.

Table 2. Maximum calcium inhibition concentrations tolerated during the duplex DNA amplification reactions.

Table 3. Recovery results for the milk samples.