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Towards Q-PCR of pathogenic bacteria with improved electrochemical double-tagged genosensing detection

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ABSTRACT

A very sensitive assay for the rapid detection of pathogenic bacteria based on electrochemical genosensing has been designed. The assay was performed by the PCR specific amplification of the *eaeA* gene, related with the pathogenic activity of *Escherichia coli* O157:H7. The efficiency and selectivity of the selected primers were firstly studied by using standard Quantitative PCR (Q-PCR) based on TaqMan fluorescent strategy. The bacteria amplicon was detected by using two different electrochemical genosensing strategies, a highly selective biosensor based on a bulk-modified avidin biocomposite (Av-GEB) and a highly sensitive magneto sensor (m-GEC). The electrochemical detection was achieved in both cases by the enzyme marker HRP. The assay showed to be very sensitive, being able to detect 4.5 ng μl^{-1} and 0.45 ng μl^{-1} of the original bacterial genome after only 10 cycles of PCR amplification, when the first and the second strategies were used, respectively. Moreover, the electrochemical strategies for the detection of the amplicon showed to be more sensitive compared with Q-PCR strategies based on fluorescent labels such as TaqMan probes.

1. Introduction

The development of rapid, inexpensive, sensitive, and high sample throughput analytical strategies, which can be used as an 'alarm' to rapidly detect the risk of contamination by food pathogens in a wide variety of food matrixes, is an intensive research area. Biosensors offer an exciting alternative to traditional methods, allowing rapid and multiple analyses that are essential for the detection of bacteria in food (Leonard et al., 2003). Moreover, biosensing strategies would be implemented in Hazard Analysis and Critical Control Points (HACCPs) systems that are, generally accepted, the most effective way to ensure food safety (Mello and Kubota, 2002). An ideal biosensing device for the rapid detection of microorganisms should be fully automated, inexpensive and routinely used both 'in field' and 'in lab'. Optical biosensors are particularly attractive as they can allow direct 'label-free' and 'real-time' detection of bacteria, but they lack of sensitivity. The phenomenon of Surface Plasmon Resonance (SPR), has shown good biosensing potential and many SPR systems are commercially available (e.g. BIAcoreTM) (Terry et al., 2005). On the other hand, electrochemically based transduction devices are more robust, user-friendly, portable, sensitive, and cost-effective analytical systems. Furthermore, electrochemical biosensors can operate in turbid media such as food matrixes (Ivnitski et al., 2000; Mehrvar and Abdi, 2004).

The detection of microbial pathogens can be achieved with nucleic acid-based detection which has shown to be more specific and sensitive than immunological-based detection. The Polymerase Chain Reaction (PCR) can be easily coupled to enhance the sensitivity of nucleic acid-based assays. By now, nucleic acid-based detection coupled with PCR has distinct advantages over culture and other standard methods such as specificity, sensitivity, rapidity, accuracy and capacity to detect small amounts of target nucleic acid in a sample. The further amplicon detection can be achieved with electrochemical DNA biosensors (Ye et al., 2003; Nebling et al., 2004; Del Giallo et al., 2005; Liu et al., 2007), reducing the time of the assay and providing results to genetic specificity. Electrochemical DNA biosensors, thus, offer a considerable promise for obtaining sequence-specific information in a faster, simpler and cheaper manner compared to traditional hybridization assays. Such devices possess great potential for numerous applications, ranging from decentralized clinical testing, to environmental monitoring, food safety and forensic investigations. However, the most

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important drawback related with electrochemical DNA biosensor is the electrochemical detection of the hybridization event in a sensitive label-free manner without the need of amplification of the genetic material.

Escherichia coli is one of the most frequent causative pathogen in human bacterial infections. Enterohaemorrhagic *E. coli* (EHEC) O157:H7 is one of the dangerous serotypes of *E. coli* that causes hemorrhagic colitis and severe hemolytic uremic syndrome, which may result in death because of acute or chronic renal failure (Karmali, 1989). Outbreaks of EHEC O157:H7 infections have been associated with food products such as ground beef (Doyle, 1991; Al-Gallas et al., 2002) and raw milk (De Buyser et al., 2001). Furthermore, documented outbreaks infections associated with fruits and vegetables have also occurred with increased frequency (Viswanathan and Kaur, 2001). In food and environmental samples responsible for disease, the levels of contamination are sometimes very low. Thus, very sensitive detection methods are required.

In this work, two different strategies for the electrochemical genosensing detection of *E. coli* were developed based on the amplification of a highly conserved sequence for several pathogenic *E. coli* serotypes. These new assays were compared in terms of specificity and sensitivity with a novel Q-PCR strategy based on TaqMan probe using fluorescence detection. TaqMan probes are oligonucleotides with a reporter dye at the 5′ end and a quencher dye at the 3′ end, which generate an increase in fluorescence emission when the reporter dye is separated from the quencher in the PCR amplification. Thus, the increase in fluorescence is a direct consequence of the amplification process (Gibson et al., 1996).

First of all, two new set of primers based on the derived consensus sequence were designed in order to produce an amplification product from several pathogenic *E. coli* serotypes by using Q-PCR procedure based on TaqMan probe. The set of primers were chosen and tested in terms of specificity and selectivity. The selected primers were labelled with biotin and digoxigenin in each extreme, in order to perform the rapid electrochemical verification of pathogenic *E. coli*. During PCR, not only the amplification of the pathogenic *E. coli* genome was achieved, but also the double tagging of the amplicon ends with (i) the biotinylated capture primer to achieve the immobilization on the genosensing transducer and (ii) the digoxigenin signalling primer to achieved the enzymatic detection through antiDIG-HRP reporter. The immobilization on the genosensing transducer was performed by using a highly specific avidin-modified biocomposite (Av-GEB) (Zacco et al., 2006a) or, in a second manner, through streptavidin-modified magnetic beads to achieve the further retention of the beads on a highly sensitive magneto sensor (m-GEC) (Erdem et al., 2006; Zacco et al., 2006b).

The results show that the combination of the genome amplification by PCR, capture of the double-tagged amplicon and electrochemical detection using a sensitive m-GEC electrode, provide promising results compared with Q-PCR based on TaqMan probes.

2. Experimental

2.1. Instrumentation

Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems). A three-electrode setup was used comprising a platinum auxiliary electrode (Crison 52–67 1, Spain), a double junction Ag/AgCl reference electrode (Orion 900200) with 0.1 M KCl as the external reference solution, and a working electrode (the GEC, m-GEC or Av-GEB electrode). Temperature-controlled incubations were done in an Eppendorf Thermomixer compact.

The magnetic separation was performed using a magnetic separator Dynal MPC-S (Prod. No. 120.20, Dynal Biotech ASA, Oslo, Norway). The PCR reaction was carried out in an Eppendorf Mastercycler Personal thermocycler.

Amplification and detection of TaqMan Q-PCR were carried out in optical-grade 96 well plates in an ABI 5700 sequence detection system (Applied Biosystems).

2.2. Chemicals and biochemicals

The graphite–epoxy composite and biocomposite were prepared using 50 μm particle size graphite powder (BDH, UK) and Epotek H77 epoxy resin and hardener (both from Epoxy Technology, USA). The Av-GEB biocomposite was prepared with avidin (Prod. No. A9275) coming from Sigma (Steinheim, Germany). The streptavidin-modified magnetic beads were Dynabeads M-280 Streptavidin (Prod. No. 112.06) and were purchased from Dynal Biotech ASA (Oslo, Norway).

Anti-Digoxigenin-POD, Fab fragments (Prod. No. 1207733) and Peroxidase biotinamidocaproyl labelled (Prod. No. P-9568) both of them used as enzyme reporters were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Sigma (Steinheim, Germany), respectively.

Two primers, 32 and 25 nucleotides long, were obtained from TIB-MOLBIOL (Berlin, Germany) and were designed for PCR amplification of *eaeA* gene fragment related to *Escherichia coli*. The primer sequences were Biotinylated *eaeA* up: 5′ bio-CTG AAT ATT CCG CAT GAT ATT AAT GGT ACT GA 3′, whose 5′ end is 228 bp from *eaeA* 5′ end, DIG-*eaeA* down: 5′ DIG-GTA ATG CAC TAT CAT CCC AGA CGA T 3′, whose 5′ end is 339 bp from *eaeA* 5′ end. The Expand High Fidelity PCR System Kit (Roche Molecular Biochemicals) was used for performing the PCR. For the TaqMan Q-PCR the same primers were used, but in this case without modifications. The TaqMan probe consisted in an oligonucleotide with a 5′ fluorescent reporter dye (FAM) and a 3′ quencher dye (NQF). The TaqMan probe sequence was 5′ FAM-ATC CAG ACC GTA TTT GC-NQF 3′, whose 5′ end is 311 bp from *eaeA* 5′ end.

All other reagents were of the highest available grade. Aqueous solutions were prepared with double-distilled water. The composition of these solutions were hybridization solution 5× SSC (0.75 mol l^{-1} NaCl, 75 mmol l^{-1} trisodium citrate, pH 7.0); Tris buffer (0.1 mol l^{-1} Tris, 0.15 mol l^{-1} NaCl, pH 7.5); blocking Tris buffer (2%, w/v BSA, 0.1%, w/v Tween 20, 5 mmol l^{-1} EDTA, in Tris buffer); phosphate buffer (0.1 mol l^{-1} phosphate, 0.1 mol l^{-1} KCl, pH 7.0).

2.3. Construction of the magneto graphite–epoxy composite (m-GEC) and avidin graphite–epoxy composite (Av-GEB) transducers

Both types of electrodes were design in our laboratories for electrochemical genosensing (Pividori and Alegret, 2005; Erdem et al., 2006; Lermo et al., 2007); and immunosensing (Zacco et al., 2006a,b). The detailed preparation of the electrodes has been extensively described by Pividori et al. for m-GEC magneto electrodes (Pividori and Alegret, 2005; Pividori et al., 2007a,b) and for Av-GEB electrodes (Zacco et al., 2006a) based on rigid graphite–epoxy composite. In the case of the Av-GEB electrode, avidin was hand mixed with the graphite power and epoxy resin paste, resulting 1% (w/w) bulk-modified biocomposite. The magneto electrodes based on GEC as well as those based on the biocomposite material were cured at 40 °C for 1 week. Before each

use, the electrode surface was renewed by a simple polishing procedure, wetted with doubly distilled water, and then thoroughly smoothed with abrasive paper and finally with alumina paper (polishing strips Prod. No. 301044-001, Orion).

2.4. Sequence selection of the eaeA gene and PCR primer design for Q-PCR with TaqMan detection and double tagging for electrochemical detection

First of all, a comparative study of *eaeA* gene of pathogenic *E. coli* was performed. The nucleotide sequences of *eaeA* gene from diverse pathogenic *E. coli* serotypes were obtained from GenBank. All the sequences ($n = 62$) from AEEC, EHEC, EPEC, STEC and ECO *E. coli* were compared using Seqman 5.0 DNA analysis software (DNA*DNAStar II, Lasergene, DNATAR Inc., USA) to determine the most conserved sequence among the serotypes of pathogenic strains. Q-PCR assays based on TaqMan probe were performed using a new set of primers designed for the *eaeA* consensus gene fragment. The electrochemical detection of the *eaeA* gene fragment was performed by the new set of primers, but in this case labelled, one of them with biotin and the other one with digoxigenin.

2.5. Q-PCR assay based on TaqMan strategy

A total of 16 strains were used by Q-PCR with TaqMan detection. Preliminary tests were carried out to determine optimum annealing temperature (60 °C and 62 °C), reaction volume (10–20 μl) and primers concentration (200 nmol l^{-1}, 450 nmol l^{-1}, 900 nmol l^{-1}). PCR reactions were performed in a reaction mixture with a total volume of 20 μl containing 9 μl of diluted DNA in sterile water (from 10 ng μl^{-1} to 1 fg μl^{-1}, in total eight 10-fold-serial dilutions). Therefore, the final concentrations ranged from 4.5 ng μl^{-1} to 0.45 fg μl^{-1}. TaqMan reactions contained 9 μl of a mixture of forward and reverse primers (final concentration 900 nmol l^{-1}) and 1 μl of TaqMan universal master mixture 2× (Applied Biosystems). TaqMan amplification conditions were carried out with an initial step at 50 °C for 2 min, and then at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 62 °C for 1 min. Negative controls were carried out using sterile water instead of DNA. Once proved the PCR amplification specificity, the selected sequence and the evaluated primers were used for the PCR amplification in the electrochemical assay.

2.6. Electrochemical characterization and potential setting

The electrodes were evaluated by cyclic voltammetry using hydroquinone as electroactive indicator in order to obtain an estimation of the optimum potential to be applied. The m-GEC and Av-GEB not-modified surfaces were compared with a GEC transducer. The applied potential window scanned by cyclic voltammetry was from −0.6 V to 1.0 V (vs. Ag/AgCl). Once the characterization was performed, the optimum potential for the electrochemical genosensing was determined by amperometry. To achieve this purpose, the electrode surface was modified with biotin-HRP. In the case of Av-GEB, the electrodes were directly placed in Eppendorf tubes containing 140 μl of biotin-HRP (20 pmol) in blocking Tris buffer. For m-GEC, streptavidin magnetic beads (6.2×10^6) were used to attach biotin-HRP (0.5 pmol) in 140 μl of blocking Tris buffer. In both strategies, the incubation step was performed with gentle shaking for 30 min at 42 °C. Then, two washing steps were performed with Tris buffer. Finally, for m-GEC strategy, the modified beads were captured on the m-GEC electrode by placing the magneto electrode inside the Eppendorf tube. In both cases, the amperometric response was determined in phosphate buffer at the following working potentials: −0.200 V, −0.150 V, −0.100 V and −0.050 V (vs. Ag/AgCl). The amperometric determination was based on the activity of the HRP enzyme as electrochemical reporter by using hydroquinone as a mediator (1.81 mmol l^{-1}) and hydrogen peroxide as the substrate for the enzyme (4.90 mmol l^{-1}).

2.7. DNA amplification and double tagging for the electrochemical detection of E. coli

Two labelled-primers with biotin and digoxigenin, respectively were used for the amplification and the double tagging of the PCR amplicon. The PCR was performed in a 100 μl of reaction mixture containing 2 ng μl^{-1} of purified DNA coming from pathogenic *E. coli* O157:H7. Each reaction contained 200 μmol l^{-1} of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 0.5 μmol l^{-1} of the double-tagging set of primers (Biotinylated *eaeA* up and DIG-*eaeA* down) and 5 U of polymerase. The reaction was carried out in buffer containing 1.5 mmol l^{-1} $MgCl_2$.

The amplification mixtures were exposed to an initial step at 95 °C for 5 min followed by cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a last step of 7 min at 72 °C. The DNA amplification was performed stopping the reaction at 5, 10, 15, 20, 25 and 30 cycles.

The same protocol was performed but varying the concentration of PCR template (from 4.5 ng μl^{-1} to 4.5 fg μl^{-1}, in total seven 10-fold-serial dilutions) in 100 μl of reaction mixture. DNA amplifications were performed stopping the reaction at 10, 15 and 20 cycles.

All of these amplifications included a blank as a control, which contained all reagents except *E. coli* template in the PCR mixture. The amplification products were analyzed by electrophoresis. As the primers were labelled with biotin and digoxigenin, the amplicon was expected to be double-tagged with both biotin and digoxigenin in each extreme, respectively.

2.8. Electrochemical detection of pathogenic E. coli

The double-tagged amplicon was detected by using Av-GEB and m-GEC electrodes. The protocol consisted of the following steps: (a) DNA amplification and double-labelling of the *eaeA* gene specific of *E. coli*; (b) immobilization of the doubly labelled amplicon by (b_i) avidin biocomposite, where the biotin extreme of the ds-DNA amplicon was attached to the surface of the Av-GEB and by (b_{ii}) streptavidin magnetic beads strategy, where the 5′ biotin end was immobilized on the streptavidin magnetic beads; (c) enzymatic labelling using antiDig-HRP able to be attached in the 3′ digoxigenin end of the amplicon; (d) magnetic capture of the modified magnetic particles by the m-GEC electrode (this step was omitted for Av-GEB strategy); (e) electrochemical determination (Fig. 1, steps a–e).

After the DNA amplification and double tagging of the *eaeA* gene, a dilution of the amplified product was prepared in 5× SSC. The PCR amplicon was incubated in 5× SSC for 15 min at 42 °C.

The immobilization of the doubly labelled amplicon was then achieved by (a) dipping the Av-GEB electrode in an Eppendorf tube containing the diluted amplicon and (b) adding 6.2×10^6 streptavidin magnetic beads in an Eppendorf tube with the diluted amplicon. In both cases, the immobilization was performed in 5× SSC solution at a final volume of 140 μl for 30 min at 42 °C.

Two washing steps were then performed with 140 μl 5× SSC for 10 min at 42 °C. After that, the enzymatic labelling was performed by using antiDig-HRP (60 μg) in blocking Tris buffer at a final volume of 140 μl for 30 min at 42 °C. Two washing steps were then performed for 10 min at 42 °C in 140 μl of Tris buffer. After the final washing step, in the case of m-GEC strategy, the modified magnetic

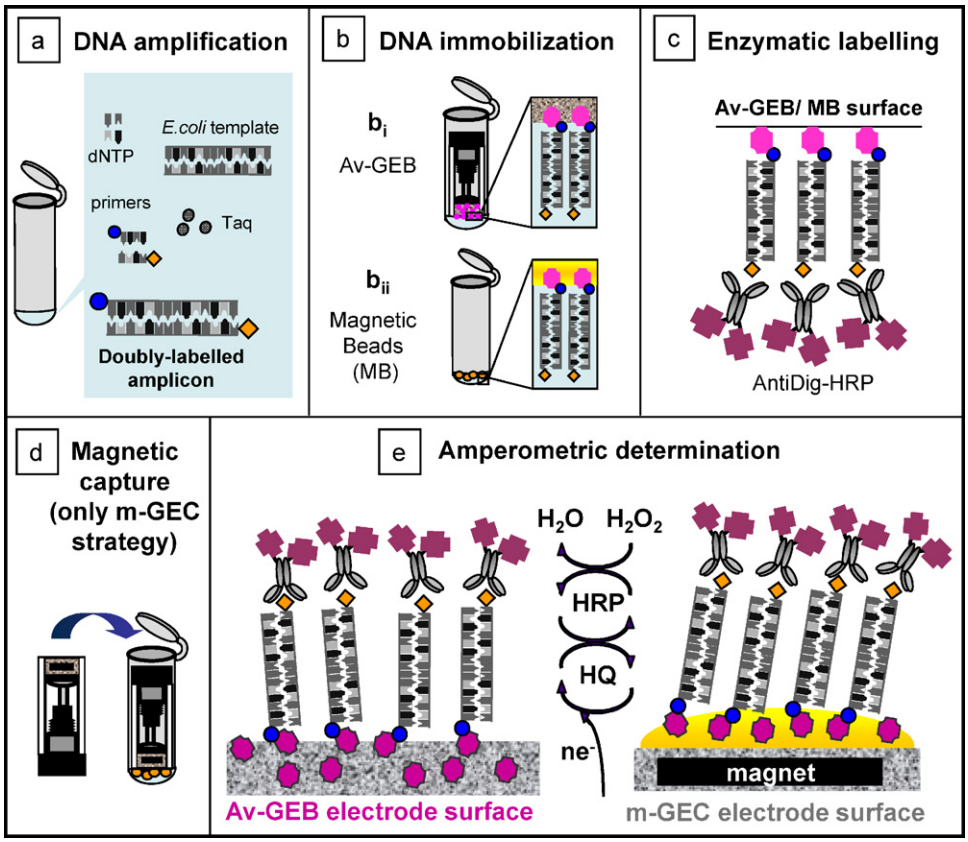

Fig. 1. Schematic representation of the electrochemical strategy for *E. coli* detection.

beads were captured by dipping the electrode inside the reaction tube.

Both genosensing strategies were used to follow the PCR amplification, stopping the reaction mixture at 5, 10, 15, 20, 25 and 30 cycles. In both cases the DNA template concentration evaluated was 2 ng μl^{-1}, but using 2.5 μl and 5 μl of the final PCR mixture, for m-GEC and Av-GEB strategies, respectively.

The detection limit of the electrochemical procedures was evaluated stopping the PCR amplification at 10, 15 and 20 cycles using 10-fold-serial dilutions of the DNA template, ranged from 4.5 ng μl^{-1} to 4.5 fg μl^{-1}.

The amperometric determination was performed as in Section 2.6. Fig. 1, step e represents the reactions that occur at m-GEC and Av-GEB sensor surfaces polarized at −0.150 V (vs. Ag/AgCl).

3. Results and discussion

3.1. Sequence selection of the eaeA gene and PCR primer design for Q-PCR with TaqMan detection and double tagging for electrochemical detection

Pathogenicity associated with EHEC involved a 94-kDa outer membrane protein called intimin-encoded by the *eaeA* gene-which has been shown to be necessary to produce the attaching-and-effacing lesions on intestinal epithelial cells (Louie et al., 1993; Ibekwe et al., 2002; Barak et al., 2005). Sequences of intimin attaching genes (*eaeA*) of pathogenic *E. coli* were compared in order to obtain a highly conserved gene sequence from which to develop PCR primers. A consensus nucleotide sequence of 469 pb was obtained (showed in the 'Supporting information' section).

A novel set of Q-PCR TaqMan primers for the amplification of *E. coli* O157:H7 as well as other *E. coli* serotypes – that would be considered human pathogens and undesirable in foods – was selected. The new set of primers was able to produce an amplification product expected to be 112 pb. The amplicon sequence was: **CTGAATATTCCGCATGATATTAATGGTACTGA**ACRCAGTACGCAGAAGATTCARTTGATCGTTAAGAGCAAATACGGTCTGGATCG**TATCGTCTGGGATGATAGTGCATTAC** where the new set of primers position is shown in bold.

The same set of primers for TaqMan Q-PCR were used for the double-tagging and electrochemical detection, but in this case, were labelled with biotin and digoxigenin, in order to achieve the immobilization and the enzymatic labelling, respectively.

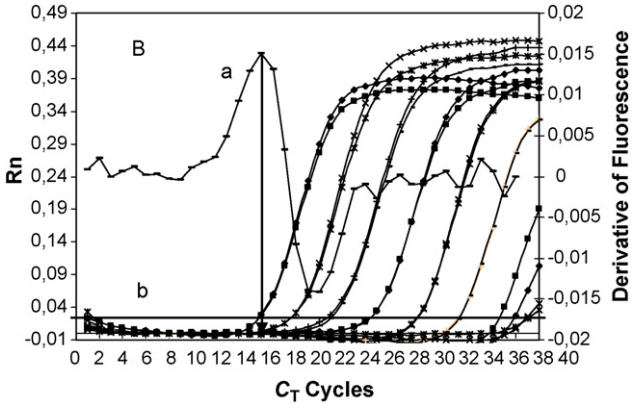

Fig. 2. Amplification plot for TaqMan strategy amplifying DNA of *E. coli* O157:H7. The parallel curves represent 10-fold DNA dilutions from 4.5 ng μl^{-1} to 0.45 fg μl^{-1}. The perpendicular to the maximum of the second derivate curve (a) to the abscissa determines the Ct for the first concentration analyzed. The threshold line (b) was after setting.

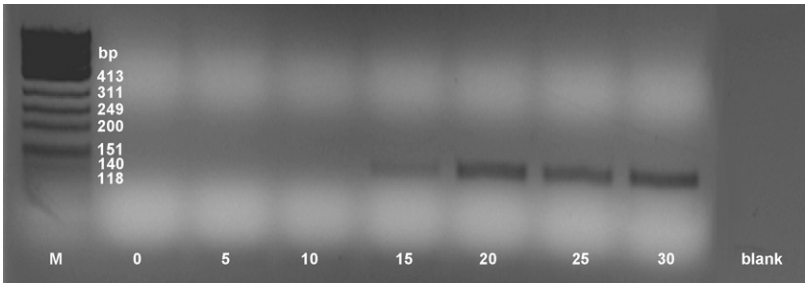

Fig. 3. Gel electrophoresis for the DNA amplification product using the double tagging set of primers.

3.2. Q-PCR assay based on TaqMan strategy

PCR experimental parameters (optimum annealing temperature, reaction volume and primer concentration) were optimized (data not shown). Real-time PCR experiments were carried out using extracted DNAs from 16 strains including pathogenic *E. coli*, non-pathogenic as well as other genera. The primers were able to amplify specifically the *eaeA* gene from all the pathogenic *E. coli* including O157:H7, O157:Neg, O29:NM, O25:NM, and O111:NM. Products for non-pathogenic *E. coli* were not detected. Fig. 2 shows the amplification plot for TaqMan strategy amplifying DNA of *E. coli* O157:H7 after optimization of experimental conditions. The parallel curves represent 10-fold DNA dilutions from 4.5 ng μl^{-1} to 0.45 fg μl^{-1}. The perpendicular to the maximum of the second derivate curve (a) to the abscissa determines the threshold cycle (Ct) for the first concentration analyzed, being the Ct the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level. The threshold line (b) was after setting. As shown in Fig. 2, Q-PCR with TaqMan strategy displays amplification curves in agreement with the DNA dilutions. A Ct value of 16 was obtained for the first DNA template and Ct increments between dilutions, ranging from 3 to 4. A linear relationship with very good fit was achieved when plotting Ct value with Log_{10} DNA concentration. The specificity of the amplification was obtained analyzing the denaturation profiles of the product and by gel electrophoresis. The melting temperatures were 72 °C. The detection limit calculated amplifying *E. coli* O157:H7, was 4.5 fg μl^{-1} for 37 cycles of PCR amplification.

3.3. Electrochemical characterization and potential setting

Av-GEB and m-GEC electrodes were submitted to cyclic voltammetry in order to obtain an estimation of the optimum working reduction potential of the mediator hydroquinone, which would be used in connection with the enzyme tracer HRP. A reduction peak was observed at −100 mV for GEC and m-GEC, while in the case of Av-GEB (1%) a slight shift in potential can be observed (−175 mV approximately, vs. Ag/AgCl), accordingly with previous results (Zacco et al., 2006a). Once this rapid characterization was performed, the optimum potential for the electrochemical genosensing was determined by amperometry using a biotinylated enzyme tracer. To achieve this purpose, the electrode surface was modified with biotin-HRP. The optimum potential was finally set in −150 mV for both genosensing strategies based on m-GEC and Av-GEB electrodes (both cyclic voltammetry and amperometric plots are shown in the 'Supporting information' section).

3.4. DNA amplification and double tagging for the electrochemical detection of E. coli

Fig. 3 shows the gel electrophoresis of the DNA amplification product using the double-tagging set of primers (0.5 $\mu mol\, l^{-1}$ of biotinylated *eaeA* up and DIG-*eaeA* down), with 2 ng μl^{-1} of DNA template. In all lanes, 5 μl of PCR reaction mixture was loaded. As shown in the picture, the PCR was successful performed with the double-tagging set of primers. Fig. 3 also shows that the double-labelled amplicon can be only observed above cycle 15 (from lanes 5 to 8, for cycles 15 to 30, respectively). No bands were observed in lanes 2, 3 and 4, loaded with the amplicon coming from 0, 5 and 10 cycles, respectively. Comparing with the results obtained with TaqMan Q-PCR (Fig. 2) the Ct cycle for 4.5 ng μl^{-1} was determined in 16 and for 0.45 ng μl^{-1} in 18.5. Thus the electrophoresis is able to detect the presence of the amplicon at lower concentration of DNA template. Agarose gel electrophoresis results also confirmed the expected size of the product (112 bp).

3.5. Electrochemical detection of pathogenic E. coli

The double-tagged amplicon was detected by using Av-GEB and m-GEC electrodes. The eCt (electrochemical threshold cycle) was

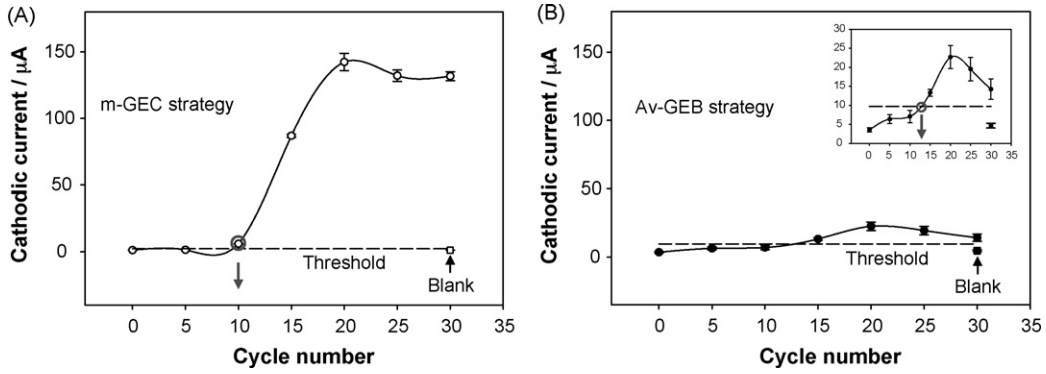

Fig. 4. Electrochemical detection of *E. coli* PCR product based on m-GEC (A) and Av-GEB (B) strategies. In all cases, 60 μg AntiDig-HRP was used. 4.90 $mmol\, l^{-1}$ of H_2O_2 and 1.81 $mmol\, l^{-1}$ of hydroquinone were also added. The medium was phosphate buffer. Applied potential = −0.15 V (vs. Ag/AgCl); $n = 3$.

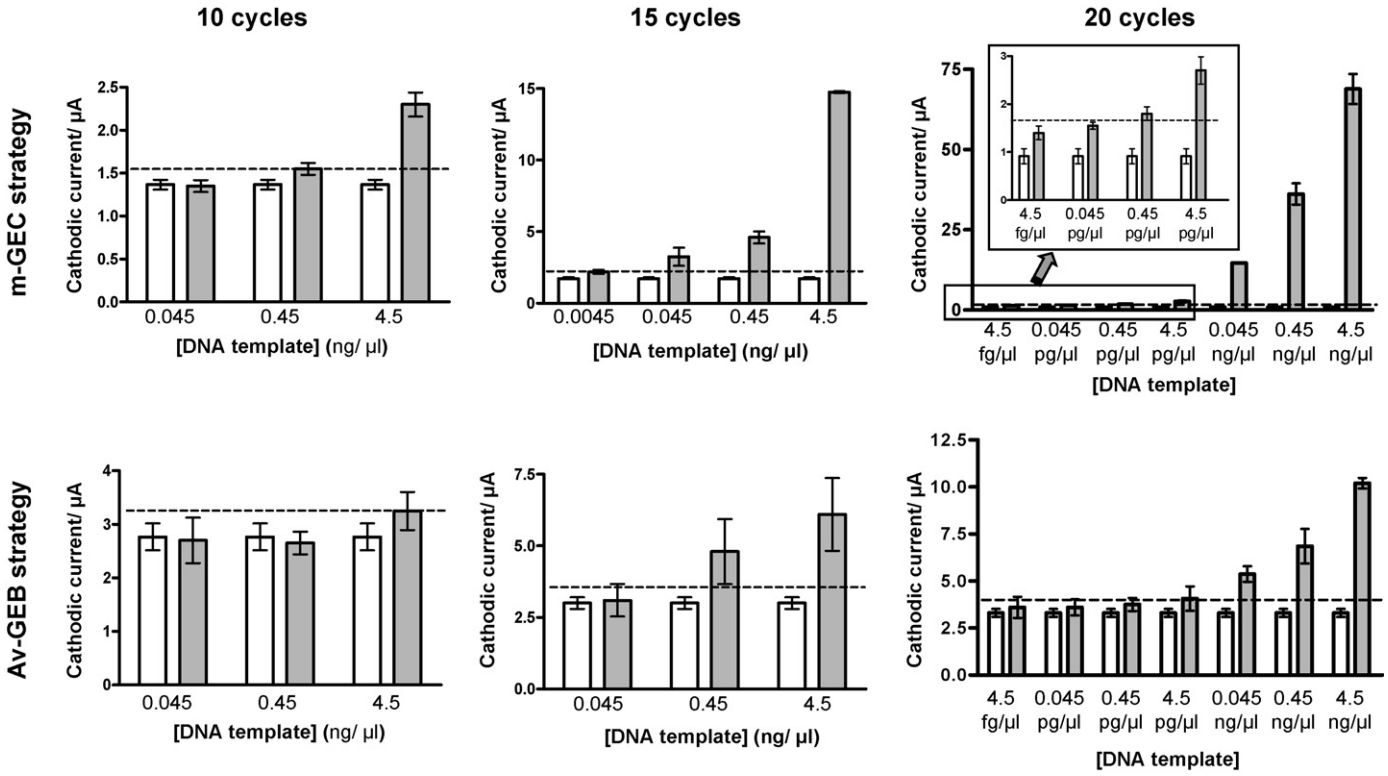

Fig. 5. LOD evaluation for m-GEC and Av-GEB strategies. PCR reactions were stopped at 10, 15 and 20 cycles. The blank controls (white bars) and the detection limit signal (discontinuous line) are shown for each strategy and PCR cycle. DNA template concentrations evaluated ranged from 4.5 ng μl^{-1} to 4.5 fg μl^{-1} (black bars). Other conditions as in Fig. 4.

determined for each strategy. The eCt is the first cycle in which there is a significant increase in the electrochemical signal above the background, taking as the background the signal value of the blank, and as the threshold, 10 times the value of the standard deviation of the background.

As shown in Fig. 4, as low as 2 ng μl^{-1} DNA template can be detected after 10 cycles (the eCt) for m-GEC (A) and 13 cycles for Av-GEB (B). Comparing the Q-PCR with electrochemical detection with both TaqMan Q-PCR (17 cycles) and gel electrophoresis (15 cycles), the genosensing strategy (based on m-GEC or Av-GEB as well) is able to detect the amplification in much less number of cycles. Moreover, it should be point out that while in the case of Q-PCR with TaqMan the volume of reaction was 20 µl, in the case of the electrochemical detection as low as 2.5 µl (m-GEC) and 5 µl (Av-GEB) where employed for the electrochemical detection.

Regarding the electrochemical genosensing strategies, m-GEC is able to detect the double-tagged amplicon in a less number of cycles than Av-GEB.

The detection limit of the electrochemical procedures was evaluated stopping the PCR amplification at 10, 15 and 20 cycles, using 10-fold-serial dilutions of the DNA template, ranged from 4.5 ng μl^{-1} to 4.5 fg μl^{-1}. In all cases 5 µl of PCR amplicon was analyzed. The electrochemical response for both electrochemical strategies is shown in Fig. 5. As can been seen, the analytical response increases with the concentration of DNA template analyzed. In the case of m-GEC strategy, detection limits of 0.45 ng μl^{-1}, 4.5 pg μl^{-1} and 0.45 pg μl^{-1} were obtained at 10, 15 and 20 cycles, respectively. For Av-GEB strategy, the detection limits at 10, 15, and 20 cycles were 4.5 ng μl^{-1}, 0.45 ng μl^{-1} and 4.5 pg μl^{-1}, respectively. As the previous results indicated, the electrochemical detection based on m-GEC strategy permits to detect low concentrations of the amplified material in relation to Av-GEB strategy.

4. Conclusions

A novel electrochemical assay for the sensitive DNA detection of pathogenic strains of *E. coli* has been developed. The assay is based on the amplification of the bacteria genome and the double tagging in order to achieve the immobilization and the electrochemical detection by using both tags.

The assay has been used for the electrochemical detection of the *eaeA* gene, related with the pathogenic activity of *E. coli* O157:H7. The biological material has been amplified by PCR using a new set of primers, which were chosen and tested in terms of specificity and selectivity by TaqMan Q-PCR procedure. The new set of primers obtained from the derived *eaeA* consensus gene fragment, was able to produce an amplification product from several pathogenic *E. coli* serotypes.

On the other hand, the new set of primers for Q-PCR with electrochemical detection were double tagged, in order to achieved the double tagging of the amplicon during PCR amplification. A unique band, as shown in gel electrophoresis and as in the case of Q-PCR with TaqMan detection, was obtained, ensuring the specificity of the assay for detecting *E. coli*.

The immobilization of the amplicon can be thus performed on avidin-modified magnetic bead followed with magnetic capture on m-GEC electrodes, or directly on avidin-modified biosensors (Av-GEB).

The use of Av-GEB electrodes provided improved features regarding selectivity of the assay by the direct interaction of the avidin surface with the biotinylated amplified material, while m-GEC strategy provided both enhanced selectivity and sensitivity by the use of streptavidin magnetic beads, which immobilized the biotinylated amplified material on the m-GEC surface and permitted a rapid magnetic separation of the unbound components.

These new strategies based on double tagging-PCR followed with electrochemical genosensing showed to be very sensitive. After only 10 cycles of PCR amplification, as low as 4.5 ng μl^{-1} and 0.45 ng μl^{-1} of original bacterial genome were detected by using Av-GEB and m-GEC strategies, respectively.

For the first time, from a consensus sequence, two primer based on different Q-PCR strategies were compared in terms of sensitivity for the amplification of pathogenic *E. coli* by using TaqMan detection and different electrochemical biosensing strategies. *E. coli* amplified material was detected by m-GEC in less number of cycles than Av-GEB strategy (10 and 13 cycles, respectively). However, the two-genosensing strategies were able to detect the amplification in much less number of cycles than TaqMan Q-PCR and gel electrophoresis (17 and 15, respectively). Moreover, the reaction volume analyzed by electrochemical strategies was four and eight times lower than in the case of TaqMan Q-PCR.

The combination of the genome amplification by double-tagging PCR, capture of the double-tagged amplicon and electrochemical genosensing detection by using the sensitive m-GEC electrode, provides a rapid, cheap and sensitive assay to quantify pathogenic *E. coli*.

Future work will be focused on multicoding genosensing of pathogenic bacteria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.02.020.

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