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Note

An electrochemical DNA biosensor for the detection of CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* in soil samples

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ABSTRACT

An electrochemical hybridization assay involving neutravidin-coated carbon screen-printed electrodes and an HRP-based detection have been shown to provide an effective tool for the genotypic analysis of extended-spectrum β-lactamase-producing *E. coli* strains in complex samples such as soil.

CTX-M (CefoTaXimase-Munich) a major type of extended-spectrum β-lactamases (EBLSs) producing *Escherichia coli* are increasingly involved in human infections worldwide (Pitout and Laupland, 2008). During the last 10 years, different reports have alerted about the dissemination of CTX-M producing *E. coli* isolates in food products, in the intestinal microbiota of healthy food-producing animals (Cortés et al., 2010; Horton et al., 2011) and in wild animals (Gonçalves et al., 2012). Recently, our group has confirmed the occurrence of CTX-M producing *E. coli* in cattle and reported for the first time the detection of such strains in farmland soils (Hartmann et al., 2012). For this purpose, standard clinical procedures based on susceptibility testing and culture methods were used as well as nucleic acid-based approaches (standard PCR, real-time PCR, gene sequencing) for the genotypic characterization of $bla_{CTX\text{-}M}$ genes. Though all of the analytical tools used during the course of this work allowed the accurate identification of ESBLs at the molecular level, faster, lower cost, easier-to-use approaches are still highly desired (Liao et al., 2006; Rubtsova et al., 2010).

In this context, electrochemical DNA biosensors offer several advantages including the ability to analyze complex matrix samples, high sensitivity, compatibility with microfabrication technology, a low power requirement, and compact instrumentation compatible with a portable device (Badihi-Mossberg et al., 2007; Lazcka et al., 2007; Tosar et al., 2010; Li et al., 2012). Earlier studies demonstrated that neutravidin-coated screen-printed carbon sensors combined with the amperometric detection of an enzyme label can be a versatile tool for the sensitive quantification of any PCR-amplified nucleic acid sequence of interest (Djellouli et al., 2007; Rochelet-Dequaire et al., 2009). The current work focuses on the use of neutravidin-coated screen printed DNA sensors for the detection of $bla_{CTX\text{-}M}$ genes from the homology group 1 in soil samples. The main steps of the hybridization procedure for the detection of amplified group 1 $bla_{CTX\text{-}M}$ sequence are illustrated in Fig. 1.

For this purpose, the primers and the Taqman (FAM TAMRA double dye) probe sequences specific for CTX-M group 1 $bla_{CTX\text{-}M}$ genes selected for the real-time PCR protocol (Hartmann et al., 2012) were used for the PCR amplification and the hybridization test, respectively. All of the primers and the probes used in this work were synthesized by Eurogentec. The development of the hybridization assay procedure was performed with soil microcosms (2 g) spiked with known and increasing amounts of *E. coli* strain EC 913SE (clinical isolate from the Bacteriology Laboratory culture collection, University Hospital of Dijon) carrying $bla_{CTX\text{-}M1}$ (1–10^6 cells per g). Microbial DNA was extracted from soil samples as previously described (Hartmann et al., 2012) and was quantified by agarose gel electrophoresis (1% agarose in TBE buffer) using calf thymus DNA dilutions as standards and the ImageQuant software (Applied Biosystems). The amplification of the 70 base-pair $bla_{CTX\text{-}M1}$ gene was carried out using the forward

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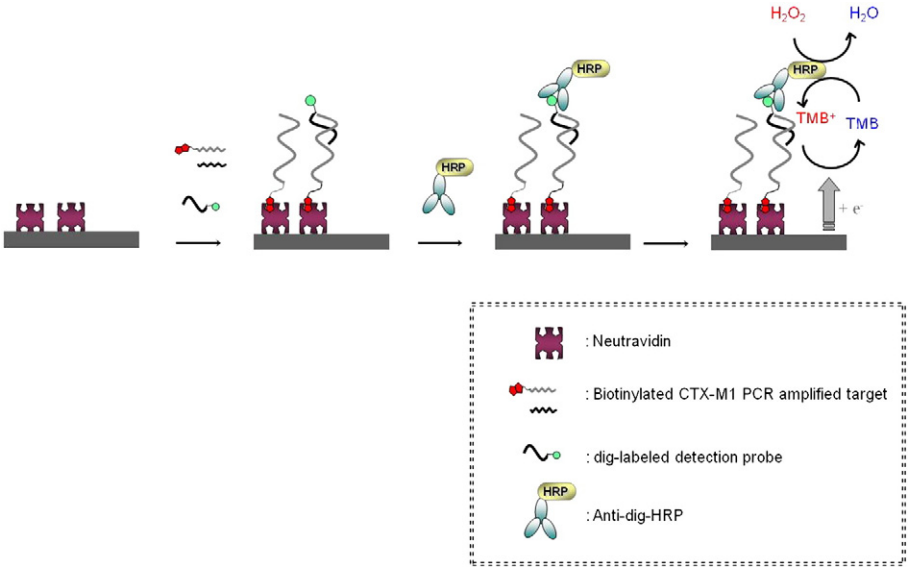

Fig. 1. Schematic representation of the HRP-based hybridization assay of $bla_{CTX\text{-}M1}$ amplified sequences on neutravidin-modified electrodes.

primer F469 (5′-CAGCTGGGAGACGAAACGTT-3′) and the 5′-biotinylated reverse primer R532 (5′-CCGGAATGGCGGTGTTTA-3′). PCR reactions were performed with 2.5 μL of the extracted genomic DNA template (10 ng) in a total reaction volume of 25 μL consisting of Taq polymerase buffer (1×), 0.25 U of Taq polymerase (MP Biomedicals), 100 μM of each of the four dNTPs (all from Qiagen) and 300 nM of each of the oligonucleotide primers. A negative control that contains all of the reagents except the DNA matrix was included in each series. Reactions were run in a PTC-200 Thermo Cycler (MJ Research). The PCR program consisted of an activation step at 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min and 74 °C for 30 min. Once the successful amplification was confirmed by agarose gel electrophoresis (2% agarose in TBE buffer) (Fig. 2a), PCR products were stored at −20 °C until the hybridization test was carried out as sketched in Fig. 1.

The biosensing platform used for this assay was prepared according to Djellouli et al. (2007) by adsorption of a monolayer of neutravidin (NeutrAvidin™ from Pierce). A 10 μL drop of a 0.5 mg mL^{-1} neutravidin solution in a phosphate buffer solution (PBS-containing 1.54 mM KH_2PO_4, 5.1 mM NaH_2PO_4, 130.9 mM NaCl, pH = 7.2) was placed onto each working electrode surface of homemade disposable arrays of screen-printed sensors and incubated for 2 h. Unless otherwise stated, all the incubations were performed at 37 °C in a water-saturated atmosphere. The amplified DNA targets were thermally denaturated at 95 °C for 10 min and cooled in an iced bath where it was mixed (1:1 v/v) with the 5′ digoxigenin-labeled $bla_{CTX\text{-}M1}$ gene specific detection probe (S490: 5′-CGTCTCGACCGTACCGAGCCGAC-3′) diluted in the hybridization buffer (Hybridowell® kit, Argene) at a final concentration of 1 μM. 7-μL droplets of the resulting mixture were applied onto the active surface of the biosensor array and incubated for 1 h. Each series of experiments included the analysis of a PCR-negative control. Next, the array was subjected to a washing cycle consisting of five rinses for 1 min in a bath of 1× washing solution (Hybridowell® kit, Argene). Following this step, 7-μL droplets containing anti-Dig-HRP (Roche Diagnostics GmbH; 1/10 dilution in PBS–BSA–Tween i.e.; PBS with 0.1% BSA and 0.1% Tween 20) were deposited on the active surface of the array and incubated for 30 min. The excess of the HRP-conjugate was washed away with PBS–BSA–Tween followed by PBS (five baths for 1 min each with each buffer). After carefully removing the rinsing solution, the amount of HRP-labeled hybrids specifically attached onto the electrode surface was determined by incubating the H_2O_2/3,3′,5,5′-tetramethylbenzidine (H_2O_2/TMB) HRP-substrate solution (v = 10 μL; Hybridowell® kit, Argene). The enzymatic reaction was allowed to proceed for 5 min after which the generated product was measured by chronoamperometry (PGSTAT12 Autolab potentiostat EcoChemie; E = −0.1 V; 90 s) by immersing in the electrochemical cell a platinum wire counter electrode and a Ag/AgCl wire reference to which all potentials are referenced. The magnitude of the cathodic current is proportional to the amount of anti-Dig-HRP anchored to the immobilized hybrids and consequently to the number of *E. coli* 913 SE CTX-M1 cells initially introduced in the soil sample. The sensitivity of the assay was investigated and the log–log calibration plot of amplified DNA resulting from soil microcosms spiked with titers of EC 913 SE $bla_{CTX\text{-}M1}$ ranging from 1 to 10^6 cells per g of soil is shown in Fig. 2b. The linearity range was extended over ca. 3 serial 10-fold dilutions i.e., ranging from 100 to 10^5 cells per g of soil. On the other hand, a quantification limit of 100 cells per g of soil was estimated which is 10 times lower than the one obtained with the real-time PCR assay for $bla_{CTX\text{-}M1}$ detection for the same soil extracted DNA (Fig. 2b and c). The fact that the electrochemical approach is more sensitive than the fluorescent detection might be explained by a partial quenching of the fluorescence signal by remaining contaminants in the soil DNA extract—without PCR inhibition—(Thakuria et al., 2008). Hence, this result clearly indicates that the use of electrochemical DNA sensors to specifically screen PCR-amplified DNA targets is an interesting alternative to real-time PCR assays to analyze extracted DNA from soils or other environmental complex samples.

While this method holds great promise for screening $bla_{CTX\text{-}M}$ genes in environmental samples, the concept of electrochemical DNA biosensors is still in the research stage. Further improvements are desired especially the development of compact, user-friendly and multichannel devices or methods for increasing the sensitivity. We have recently shown a thin layer-based amperometric enzyme detection concept both able to improve the sensitivity of the assay and to reduce the assay time under 30 min (Rochelet et al., 2012).

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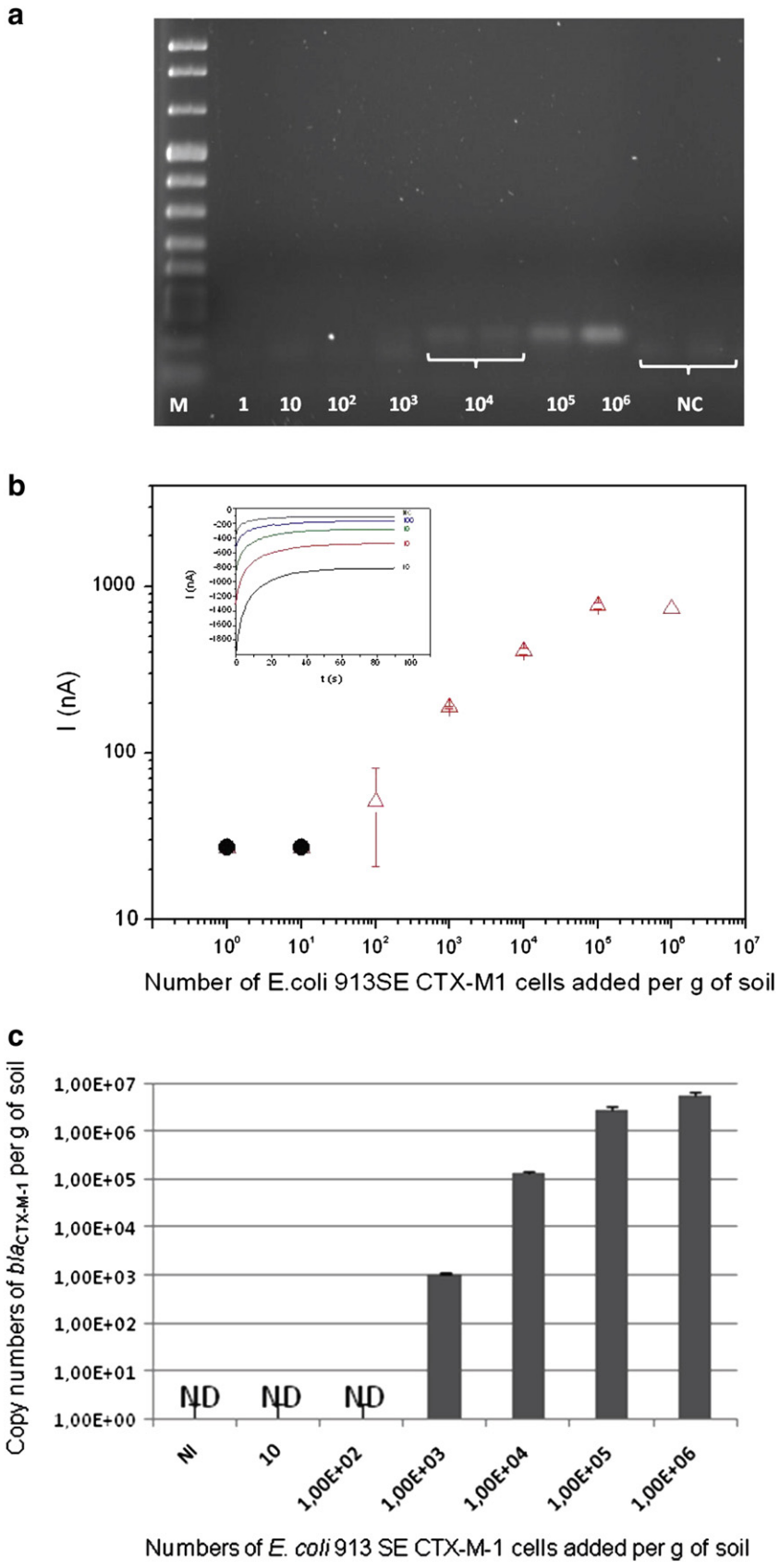

Fig. 2. (a) Gel electrophoresis image of $bla_{CTX\text{-}M1}$ biotinylated PCR products obtained from soil microcosms spiked with increasing amounts of *E. coli* strain 913SE CTX-M1. M and NC correspond to the molecular weight marker (Eurogentec) and the negative control, respectively. (b) Log–log plot of the absolute value of the chronoamperometric steady state current measured at t = 70 s after the negative control signal subtraction *vs.* increasing known amounts of CTX-M1 *E. coli* strains added in soil samples. Inset: set of chronoamperometric curves recorded at the DNA sensor (E = −0.1 V *vs.* Ag/AgCl) after a 5 min-incubation with the HRP substrate solution. (c) Detection levels of $bla_{CTX\text{-}M1}$ genes by real-time PCR from DNAs extracted from the same spiked soil microcosms. ND, not detected. For the hybridization and real-time PCR methods, error bars represent the standard deviation of two replicates.

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