Amperometric detection of extended-spectrum β-lactamase activity : application to the characterization of resistant E.coli strains

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Amperometric detection of extended-spectrum β-lactamase activity: application to the characterization of resistant *E. coli* strains

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The amperometric detection of extended-spectrum β-lactamase (ESBL) with carbon screen-printed sensors was investigated in the presence of the Nitrocefin, a commercially-available β-lactamase chromogenic cephalosporin substrate. Using an ESBL isolated from a clinical sample, it was shown for the first time that the intensity of a specific anodic pic current (E_P = ~+0.3 V *vs.* Ag/AgCl) resulting from the catalytic hydrolysis of the β-lactam ring was proportional to the amount of ESBL. The proof-of-principle of a novel susceptibility assay for the rapid and accurate identification of ESBL- producing bacteria was then demonstrated. The detection scheme relied on (i) the culture of the sample in a medium containing the cefotaxime supplemented or not with the clavulanic acid inhibitor to allow the specific determination of ESBL producers (ii) followed by the incubation of the bacteria with the Nitrocefin and (iii) the measurement of the enzyme product by cyclic voltammetry. The amperometric assay was further applied to the characterization of *E. coli* strains and to the quantification of the ESBL producers. A detection limit of 5×10^4 cfu mL^{-1} ESBL-producing *E. coli* was achieved after a 10 min incubation time. In contrast to the approved routine assays, the electrochemical approach, which did not require isolated colonies to be performed, provided quantified results regarding ESBL activity within a few hours. Finally, owing to its cost-effectiveness, portability and simplicity, this test holds great promise for clinical and environmental applications.

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1. Introduction

β- lactamases are the main cause of bacterial resistance to penicillins and cephalosporins owing to their ability to catalytically cleave the β-lactam ring before the antibiotic can kill its target bacteria.[1] The production of extended-spectrum β-lactamases (ESBLs) is one of the most significant mechanisms of resistance to oxyimino cephalosporin antibiotics (*i.e.*, cefotaxime, ceftazidime, ceftriaxone) among *Enterobacteriaceae*.[2] However, ESBLs do not hydrolyze the cephamycins, the carbapenems and their hydrolytic activity can be inhibited by several β-lactamase inhibitors such as clavulanic acid and tazobactam.[3] A variety of ESBLs, mostly of the CTX-M, TEM, and SHV types, have been reported in *Enterobacteriaceae*.[4] Accurate identification ESBL-positive *Enterobacteriaceae* in due time is crucial for optimal patient management as well as for immediate institution of appropriate infection control measures to prevent the spread of such microorganisms.[5] Current routine techniques for detecting ESBL producers are culture-based or molecular methods.[6] The simplest approach relies on the determination of antibiotics susceptibility and inhibition of bacterial growth by cephalosporin antibiotics in the presence of inhibitors. The double-disk synergy test and the ESBL 'Etest'[7] as well as semiautomated microbiology systems (VITEK2 System; Phoenix Automated Microbiology System) were proposed for that purpose.[8] Despite their cost effectiveness, all these tests require overnight growth, meaning that up to 24 to 48 h can elapse before ESBL production is detected once the isolate has grown. Such a delay seriously hampers the rapid initiation of an appropriate antibiotic therapy. Moreover, the interpretation of the results can be complicated especially for multi antibiotic resistance and associated with falsely negatives. Alternatively, resistance can be analyzed on genotypic level by using molecular-biological methods such as PCR and sequencing,[9] real-time PCR,[10] DNA microarrays[11] (Checkpoints). Although they can provide specific characterization of the ESBL genes, all these PCR-based assays are very expensive,

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time-consuming and usually restricted to epidemiologic surveillance or refined antimicrobial therapy.[12] All in all, simple and efficient methods for the fast identification of ESBLs are still needed.

Towards this end, flow cytometric,[13] mass spectroscopic (MALDI-TOF)[14] and colorimetric microtiter plates[15] assays have been recently described. For example, Nordmann *et al.*[15] reported a biochemical test for the rapid identification of ESBLs in *Enterobacteriaceae*. Since the hydrolysis of the β-lactam ring of a cephalosporin yields a carboxylic acid group and thus can lead to the acidification of unbuffered solutions, it was shown that ESBL activity can be linked to a color change mediated by a pH indicator (red phenol). Despite its low cost and its rapidity (less than 1 h), this assay still required a time-consuming step to growth and to isolate the bacteria. On the other hand, although positive and negative controls are run in parallel, acidification of the medium can occur for reasons other than ESBLs action. Another much more specific chemical ESBLs detection approach relies on the use of chromogenic cephalosporins, whose hydrolysis can be directly monitored in the visible wavelength range.[16] Such tests have been mainly used for the kinetic characterization of β-lactamases and the detection of the enzymes in crude extracts and chromatographic fractions. A rapid assay (Cica β-Test) based on paper strips is also commercially available for the detection of ESBLs producers.[17] Nevertheless, this technically simple and rapid (15 min) method still requires the use of isolated colonies to avoid misinterpretation during the colorimetric detection, meaning that the ESBLs strains are not identified until 24 h after a clinical specimen is taken.[18] In view of this limitation, it is therefore highly desirable to investigate alternative assays that do not require the need of isolated colonies and thus shorten the result delay.

Owing to their advantages over colorimetric and spectrophotometric techniques *i.e.*, simplicity, sensitivity, use of disposable screen-printed sensors with small volumes of sample, field-portable instrumentations and measurements can be performed in turbid media, electrochemical methods are highly suitable for the reliable and the specific characterization of microorganisms in complex medical, environmental and food samples.[19]

With the aim to propose an electrochemical method for the rapid detection of β-lactamase activity in bacterial suspensions, the voltammetric behavior of the widely used and commercially available chromogenic antibiotic cephalosporin named Nitrocefin was investigated. ESBLs were able to convert the Nitrocefin substrate into a chromogenic compound but also into an electroactive product, which could be specifically determined by cyclic voltammetry with disposable carbon screen-printed electrodes. The use of disposable SPEs allowed for simplified operation in microliter solutions and greatly reduces the costs. The Nitrocefin-based electrochemical detection was finally applied the identification and quantification of an ESBL-producing *Escherichia coli* (*E. coli*) strain.

2. Materials and methods

2.1. Reagents and solutions

Phosphate-buffered saline (PBS; 100 mM; pH = 7.0) and all of the solutions were prepared with Milli-Q 18-MΩ water (Millipore purification system). Cefotaxime sodium salt and potassium clavulanate were obtained from Sigma. Nitrocefin was purchased as a pure powder from Merck Millipore and working solutions were prepared as described by Livermore and Brown.[20] Briefly, a 10^{-2} M stock solution was prepared by dissolving the Nitrocefin powder in dimethylsulfoxide and aliquots were stored at −20 °C. As Nitrocefin is light sensitive, 0.5 mM working solutions were daily prepared by diluting the stock solution in PBS. A β-lactamase (ESBL) enzyme extract was prepared from *E. coli* strain 1485 (Laboratory of Bacteriology University Hospital of Dijon) known to carry a CTX-M-1 type ESBL (*bla* gene sequencing).[21] In the absence of commercially available ESBLs, this enzyme extract was used as a standard in this study. Culture media, consisting of Luria broth (LB; 10 g L^{-1} bacto tryptone, 5 g L^{-1} bacto yeast extract, 5 g L^{-1} NaCl); of Tryptone salt (TS; 10 g L^{-1} bacto tryptone, 5 g L^{-1} NaCl, pH = 7) and of TBX agar plates (35.6 g L^{-1} Tryptone Bile X-Glucose) were home-made. Cefotaxime and potassium clavulanate stock solutions (1 mg mL^{-1}) were freshly prepared in PBS before their addition to LB and TBX media. All media and plastic/glassware were sterilized by autoclaving at 120 °C for 15 min.

2.2. Apparatus and electrodes

All of the electrochemical measurements were carried out with a PGSTAT12 Autolab potentiostat (Metrohm) interfaced to a PC system with GPES version 4.9 software. A manual screen-printer (Circuit Imprimé Français) was used to produce disposable screen-printed sensors. Arrays of six sensors were printed with a carbon ink (Electrodag® PF 407A, Acheson Colloids) on a polyester flexible film, each of them consisting of a disk-shaped working electrode (7.07 mm^2) and a half ring counter electrode with conductive tracks for the electrical contacts. Each electrochemical cell was delimited with an adhesive ring and connected to the potentiostat through a commercial connector (Dropsens). Cyclic voltammetric measurements (CV; scan rate of 50 mV s^{-1}) were performed at room temperature with a working volume of 20 μL in which was dipped an Ag/AgCl wire reference electrode.

2.3. Amperometric and colorimetric determination of ESBL activity with the Nitrocefin

To 5 μL of the ESBL solution – dilutions of the crude extract in PBS ranging from pure to 1/256 ratio – were added 45 μL of the 0.5 mM Nitrocefin solution in PBS in a polypropylene tube. All the reaction mixtures were incubated for 10 min at room temperature in the dark. The water-soluble and red electroactive product generated was then determined by both CV and absorption spectrophotometry. Each set of experiments included a negative control containing all of the reagents except the β-lactamase. In the case of the electrochemical approach, the incubation step was followed by transferring a

20-μL droplet of the reaction mixture onto the surface of a carbon sensor and the enzymatic product was measured by cyclic voltammetry as described above. The intensity of the anodic peak current (E = ~+ 0.3 V *vs.* Ag/AgCl) was taken as the analytical response. The colorimetric detection was performed by successively diluting the solutions in PBS (5-times), transferring the resulting solutions in single-use cuvets (ratiolab® Q-VETTES semi-micro, n° 2712120) before reading their optical density at λ = 520 nm with a DU® 800 spectrophotometer (Beckman Coulter). Data shown are the average from two independent measurements after blank subtraction.

2.4. Preparation of bacterial cultures

Two *E. coli* strains obtained from the INRA collection were used in this study: an ESBL-producing strain from cultivated soil (*E. coli* 6690) and a non-ESBL producer (*E. coli* K12 6395). Those strains had previously been characterized for their β-lactamase content at the molecular level.[10b] Aliquots of both *E. coli* stock cultures were maintained in a 50% glycerol solution at −20 °C and handled according to rules appropriate to biosafety level 2 microorganisms. *E. coli* cultures were grown in LB media at 37 °C for 4h30 until they reached the logarithmic phase (~2×10^8 cfu mL^{-1}). The resulting bacterial cultures were spectrophotometrically monitored (λ = 600 nm), serially diluted (10-fold steps) in TS, and 100-μL aliquots appropriate dilutions were applied to TBX agar plates and then incubated for 24 h at 37 °C for enumeration of colonies. For ESBLs resistance testing, LB and TBX media were supplemented with cefotaxime (4 μg mL^{-1}) associated or not with the potassium clavulanate (10 μg mL^{-1}).

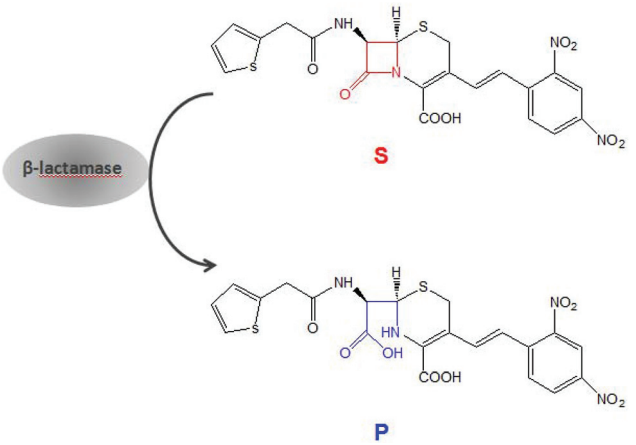
2.5. ESBL amperometric assay using cultivated *E. coli* strains

The qualitative voltammetric characterizations were performed after centrifugation (7000*g*; 10 min) of 1 mL of the above log-phase cultures. After removing the supernatant, a 50 μL-volume of a 500 μM Nitrocefin substrate solution in PBS was incubated in the dark for 10 min. For the quantitative analysis, the cultures were further diluted to concentrations ranging from 10^3 to 10^7 cfu mL^{-1} in 30 mL of TS medium. A 10 mL volume of each bacterial suspension was then filtered through 0.45 μm filter (HVLP, 13 mm, Millipore) using a syringe and membrane filter holder (Swinnex®, 13 mm, Millipore). The resulting membrane filter was placed into a polystyrene microwell (24 well plate, Cellstar®, 662160) containing 80 μL of a 500 μM Nitrocefin substrate solution in PBS. After enzyme incubation in the dark for 10 min, voltammetric measurements were carried out as described above. Two different sets of parallel experiments were conducted for each dilution.

3. Results and discussion

3.1. Electrochemical characterization of Nitrocefin hydrolysis

The commercially-available chromogenic cephalosporin antibiotic known as Nitrocefin has been the predominant assay substrate used for the detection of β-lactamase activity.[16]



Scheme 1 Hydrolysis of the β-lactam ring of the Nitrocefin (S) by a β-lactamase.

During the catalytic hydrolysis of the β-lactam ring (Scheme 1), the yellow Nitrocefin substrate (S) is converted into a red product (P) which produces a striking visual color change. The strong conjugation of both the β-lactam ring and the dihydrothiazine ring with the dinitrostyrene group leads to a significant bathochromic shift of λ_{max} which allows quantitative measurement by absorption spectroscopy.[16a] To the best of our knowledge, the electrochemical detection of Nitrocefin has never been reported in the literature. Hence, the electrochemical behaviors of S and P were first investigated. For this purpose, Nitrocefin was enzymatically hydrolyzed using an excess of ESBL and Fig. 1 compares the resulting cyclic voltammograms (CVs) recorded on a SPE in PBS. Nitrocefin and its hydrolyzed form both exhibited an irreversible anodic oxidation peak (a_1; ~+1.0 V) which might be attributed to the oxidation of the thiol group in the dihydrothiazine group or to another function specific to cephalosporin derivatives since similar peaks were also observed for cefotaxime and ceftazi-

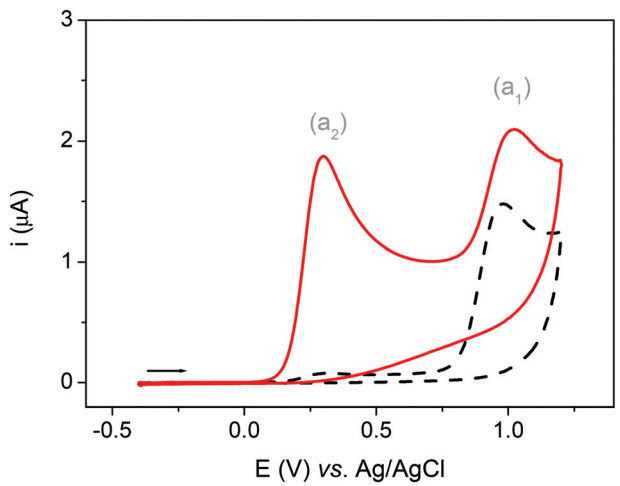


Fig. 1 Cyclic voltammetric curves (v = 50 mV s^{-1}) of a 500 μM solution of Nitrocefin (---) alone and (—) after a 10-min incubation with the ESBL (40-fold dilution of the stock solution) at a SPE.

dime (data not shown). One more well-defined irreversible oxidation wave (a_2) was observed for the catalytic product P at much lower anodic potential (+0.3 V). The amino substituent resulting from the disruption of the β-lactam ring probably reduces the oxidation potential of initially inactive electron-rich groups of Nitrocefin. Though further investigations are still required to fully understand the electrochemistry of both S and P molecules, the magnitude of the anodic peak current a_2 corresponding to the specific oxidation of P was selected as the analytical response to monitor the β-lactamase activity. Finally, as shown on the Nitrocefin CV, a small residual signal can be observed at +0.3 V owing to the light-sensitivity of the Nitrocefin. Consequently, fresh solutions were daily prepared and incubated in the dark for all of the experiments.

3.2. Measurements of the ESBL activity

Since the use of Nitrocefin as chromogenic substrate has been widely reported for detection, inhibition and kinetic studies of β-lactamases, we took advantage of the literature to select our experimental conditions *i.e.*, incubation of a 500 μM nitrocefin solution in 100 mM PBS (pH = 7) in the dark at room temperature for 10 min.[20] With the aim of a comparative study, the ESBL Nitrocefin assay was performed using both the spectrophotometric and the amperometric detection. Each series of experiments was carried out by varying the ESBL concentrations and the log–log calibration plots of ESBL are shown in Fig. 2 for both the electrochemical (curve A) and colorimetric (curve B) approaches. Each measurement was normalized to the signal obtained in the absence of ESBL (substrate blank response). The comparison of the calibration plots indicated that the electrochemical approach allowed the quantitative detection of the ESBL activity with a sensitivity close to the one of the absorption spectroscopic method. Nevertheless, owing to the Beer–Lambert law deviation for high concentrations, a wider linear range was obtained for the amperometric detection. It is also worth noting that the reproducibility of both the electrochemical and the spectrophotometric measurements carried out in duplicate were similar.

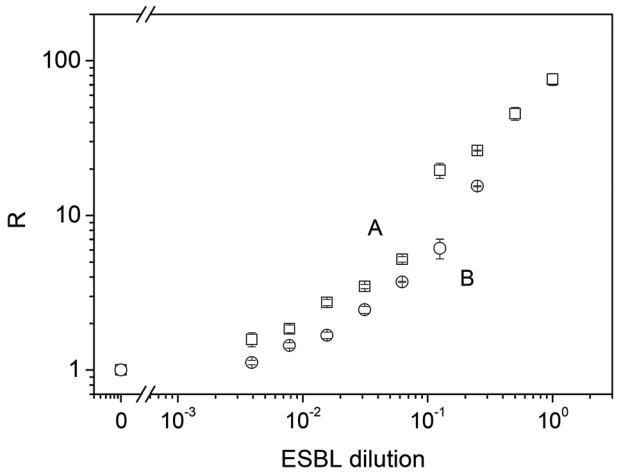


Fig. 2 Log–log calibration plots of ESBL for (A, □) the electrochemical- and (B, ○) the spectrophotometric-based assays. The analytical responses were obtained after a 10-min incubation time with a 500 μM Nitrocefin solution prepared in PBS. The solutions were 5-times diluted in PBS to perform the colorimetric detection. The *R* values correspond to the current responses and optical densities normalized to the zero ESBL concentration (i_0 = 50 ± 5 nA; OD_0 = 0.097 ± 0.008). The error bars represent the standard error of two measurements.

3.3. Specific amperometric detection of ESBL-producing *E. coli* strains

To evaluate the ability of the amperometric approach to detect ESBL producing bacteria – *i.e.*, able to catalytically hydrolyze cephalosporins -, two *E. coli* strains with well characterized genotypes (see section 2.4) including one ESBL producer ($ESBL^+$) and another one known to be non ESBL producing ($ESBL^-$) were selected. A series of preliminary qualitative experiments was carried out by raising both strains in LB media containing 4 μg. mL^{-1} of the cefotaxime antibiotic. After a centrifugation step, the bacterial pellets were incubated with the Nitrocefin substrate according to the previously selected experimental conditions. Fig. 3 displays the CV responses obtained for the $ESBL^+$ (curve A) and the $ESBL^-$ (curve B) strains. As expected, the curve (A) showed the anodic peak recorded (~+0.4 V *vs.* Ag/AgCl) associated to the catalytic hydrolysis of the nitrocefin β-lactam ring by the $ESBL^+$ strain whereas no signal was recorded for the non-ESBL producer (curve B). This result was in good agreement with the optical density values of 0.934 and 0.002 obtained for the $ESBL^+$ and $ESBL^-$ strains, respectively. The culture media supplemented or not with the antibiotic were also submitted to the same entire protocol. The absence of nonspecific signal in the [0.2–0.6 V] potential range on the resulting CVs (data not shown) confirmed that both the LB media and the cefotaxime antibiotic did not hamper the amperometric detection. As Nitrocefin can be hydrolyzed by any β-lactamase, it should be

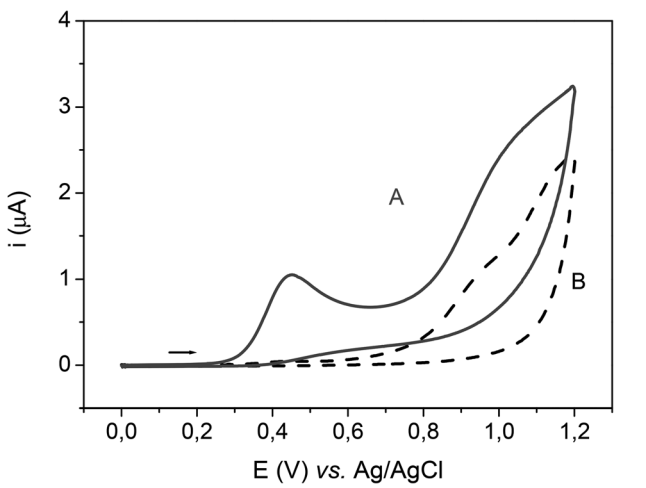


Fig. 3 CV curves (v = 50 mV s^{-1}) recorded at a SPE for (A) the $ESBL^+$ and (B) the $ESBL^-$ *E.coli* strains raised in a culture media containing 4 μg mL^{-1} cefotaxime at 37 °C for 4h30 after centrifugation (1 mL; 7000*g*; 10 min) and incubation of the pellet with 50 μL of a 500 μM Nitrocefin solution for 10 min.

noted that metallo-β lactamases-producing strains which are as well able to grow in the presence of cefotaxime will also give a positive amperometric result. Taking into account the definition of ESBL positivity based upon growth assessment in the presence of cephalosporins with and without clavulanic acid, the specific identification of ESBL producers was performed by simultaneously growing each strain in two separate liquid media containing cefotaxime either alone or supplemented with the potassium clavulanate inhibitor. Once the bacteria recovered, the further steps of the Nitrocefin test proceeded as described above. The CV curves recorded during the analysis of the strain harboring ESBL are shown in Fig. 4. The characteristic anodic peak resulting from the hydrolysis of the Nitrocefin was observed for the strain raised with cefotaxime (curve A) while no signal was recorded for the culture achieved in the presence of potassium clavulanate (curve B), thus confirming an ESBL positive result. Our measurements were also in concordance with the *E. coli* enumeration values of 80 cfu mL^{-1} and 1.8×10^8 cfu mL^{-1} found for the cultures supplemented or not with the inhibitor, respectively. The ability of the method for the quantitative determination of ESBL producers was finally assessed by diluting the $ESBL^+$ *E. coli* culture raised in cefotaxime over the 5×10^4 to 5×10^7 cfu mL^{-1} range. The curve shown in Fig. 5 exhibited a large linearity range (over more than to 2 decades) which allowed quantitative determination of ESBL producers to be envisaged. The plateau observed at high $ESBL^+$ concentrations suggested that the Nitrocefin substrate was almost completely hydrolyzed. Although the detection limit strongly depends on several experimental parameters – such as the growth time, the culture volume filtered to perform the assay, the incubation time with S – a value of the 5×10^4 cfu mL^{-1} $ESBL^+$ strains was estimated for our selected experimental conditions. As expected from the enumeration result (70 cfu mL^{-1}), the signal obtained (open square) for the $ESBL^+$ strain grown in the medium supplemented with cefotaxime plus potassium clavulanate was very close to the background signal of S (open circle). Interestingly, the reported Nitrocefin-based amperometric assay, which did not require isolated colonies to be performed, provided easy-to-read quantitative results and thus could overcome the limitations of the current colorimetric and susceptibility tests.[16–18] Moreover, with only a few hours of incubation with cefotaxime supplemented or not with potassium clavulanate (at least until the exponential phase is reached *i.e.*, 6 h) before carrying out the amperometric assay (15 min), the method allowed to deliver the results regarding the ESBL activity on the same day. Though further validations are necessary before its diagnostic use, this inexpensive assay might also be useful in the pharmaceutical area to speed up the development of new β-lactam antibiotics and β-lactamase inhibitors. Finally, since any other antibiotic and/or inhibitor associations can be used during the first step, this assay may allow the identification of other groups of β-lactamases.

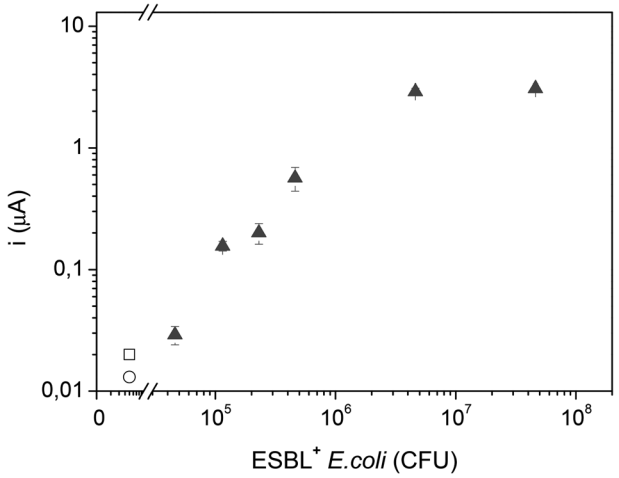


Fig. 5 Amperometric quantification of the $ESBL^+$ strain after its culture in a medium supplemented with 4 μg mL^{-1} cefotaxime at 37 °C for 3h30. The culture was then serially diluted and 10 mL-volumes were filtered (∅ = 13 mm; 45 μm) in duplicate. The $ESBL^+$ amount corresponds to the number of bacteria recovered after filtration and incubated with 80 μL of the 500 μM Nitrocefin solution for 10 min. Error bars represent the standard deviation of two measurements. Open square symbol: control with the $ESBL^+$ strain raised with 4 μg mL^{-1} cefotaxime acid and 10 μg mL^{-1} of clavulanic acid. Open circle symbol: Nitrocefin background signal.

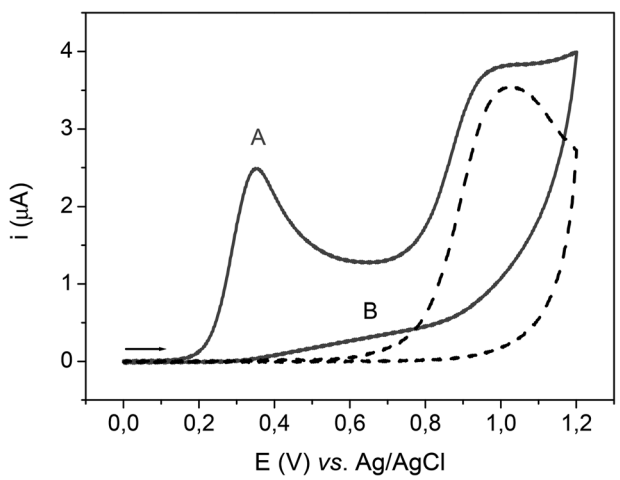


Fig. 4 CV curves (v = 50 mV s^{-1}) recorded at a SPE for the $ESBL^+$ *E. coli* strain raised in a culture media containing 4 μg mL^{-1} cefotaxime (A) alone or (B) supplemented with 10 μg mL^{-1} potassium clavulanate acid after centrifugation (1 mL; 7000*g*; 10 min) and the 10-min incubation step of the pellet with 50 μL of a 500 μM Nitrocefin solution.

4. Conclusions

The indirect electrochemical determination of ESBL activity could be achieved with disposable screen-printed sensors using the Nitrocefin substrate and was successfully applied to the identification of ESBLs producers. Since Nitrocefin is sensitive to hydrolysis by all known β-lactamases produced by Gram-positive and Gram-negative bacteria, a rapid two-step specific protocol - performed in less than a working day – was

proposed: (1) the growth of the *E. coli* strains with the cefotaxime supplemented or not with the potassium clavulanate inhibitor for a few hours followed by, (2) the incubation of the recovered bacteria with the Nitrocefin substrate which hydrolysis could be monitored by amperometry. While offering the same specificity as the approved routine procedures, the detection scheme described here had also its own advantages of simplicity, rapidity and quantitative analysis. This approach has recently allowed the specific detection of bacterial species with various types of β-lactamases such as ESBLs, penicillinases, cephalosporinases (Betelli *et al.*, submitted for publication). Finally, the analytical performances of the reported method are currently assessed on clinical blood culture samples.

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