Talanta 174 (2017) 507–513

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Electrochemical magneto immunosensor based on endogenous β-galactosidase enzyme to determine enterotoxicogenic *Escherichia coli* F4 (K88) in swine feces using square wave voltammetry

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ARTICLE INFO

Keywords:
Escherichia coli F4 (K88)
Swine stool samples
Electrochemical Immunosensor
Square wave voltammetry

ABSTRACT

Diseases caused by enterotoxicogenic *Escherichia coli* F4 (K88) (ETEC F4) are a problem in swine production establishments. Due to the high rate of mortality and morbidity of *E. coli* infections, a rapid and accurate diagnosis is important in order to choose an appropriate treatment to reduce the economic impact. Therefore, an electrochemical magneto-immunosensor (EMI) was developed to detect and quantify ETEC F4 in swine feces samples through a direct non-competitive immunoassay. ETEC F4 was selectively captured by immunomagnetic separation. The detection principle was based on the activity of β-galactosidase endogenous enzyme (β-gal), which hydrolyses the p-aminophenyl-β-D-galactopyranoside (p-APG) producing p-aminophenol (p-AP), which was oxidized on a carbon screen printed electrode (CSPE) using square wave voltammetry (SWV). All parameters related to construction and electrochemical responses were optimized. The total analysis time to quantify ETEC F4 using the EMI was less than 2 h and the limit of detection (LOD) was 33 CFU mL^{-1}. The perceptual relative error ($\%E_r$) was 20%. The magneto-immunosensor was validated versus conventional method of culture and plate count, obtaining a very good agreement. The EMI is simple, fast and economical to detect and quantify ETEC F4 in swine feces samples, being thus a valuable tool in swine production.

1. Introduction

Colibacillosis produced by enterotoxigenic *Escherichia coli* F4 (K88) (ETEC F4) is a common bacterial disease that affects swine in the neonatal and post-weaning periods. This disease produces diarrhea and dehydration, causing significant economic losses due to the high rate of mortality and morbidity [1].

The ETEC F4 bacteria is develops in swine environment when hygienic conditions are inadequate [2], and it survives for several months in swine farms, spreading rapidly. Thus, pigs can become infected via fecal-oral contact with other pigs, food, water or sites contaminated with bacteria.

The diagnosis of neonatal and post-weaning colibacillosis is based on clinical observations, culture techniques and identification of the main virulence factors, i.e., fimbriae and enterotoxins, using phenotypic or genotypic methods [3–5].

In veterinary practice, when the first clinical signs of enteric infections appear, it is made presumptive diagnosis is, and it is started an antimicrobial treatment. However, the use of antimicrobials from different families leads to the development of resistance against *E. Coli* [1]. Thus, there is a potential danger of transferring genes resistant to antimicrobial agents used in the food chain [6]. The presence of ETEC F4 produces an import economic impact on swine breeding establishments. Thus, it is necessary a rapid identification of the presence of ETEC F4. ETEC F4 detection can be carried out in samples of intestinal tissues or feces of swine with diarrhea [7]. ETEC F4 must be isolated by selective culture medium and characterized by biochemical tests. For this purpose, conventional immunological techniques such as agglutination, and ELISA [8–10] or molecular techniques such as PCR [11–14] are used to identify virulence factors. PCR has remarkable advantages over culture techniques and other standard techniques such as specificity and sensitivity. However, the PCR direct application

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http://dx.doi.org/10.1016/j.talanta.2017.06.059
Received 26 April 2017; Received in revised form 19 June 2017; Accepted 20 June 2017
Available online 21 June 2017
0039-9140/

in samples such as swine feces is limited. It requires a prior isolation of ETEC F4 using a selective medium or commercial kits to extract the bacteria from the DNA [15]. Therefore, common methods used for the bacteria detection in clinical samples have limitations in terms of time, costs and complexity [16].

Thus, it is necessary to have simple, selective and reliable methodologies that allow a rapid diagnosis of ETEC F4. The development of electrochemical immunosensors has gained importance in recent years since they are devices of easy design, economic, in some cases disposable, miniaturizable and very sensitive [17–19]. Screen printed electrodes have great advantages such as they can be used outside the laboratory and are disposable, desirable features when working with pathogenic substances.

On the other hand, immunosensors have attracted a great interest in the field of Analytical Chemistry due to their quick responses, sensitivity, and high selectivity to determine a given substrate. In the construction of immunosensors, the immobilization of antibodies onto the solid surface is a key step, which determines the stability, reproducibility and sensibility of the immunoassay [20]. The use of magnetics beads (MBs) coated covalently with Protein G confers specific binding and orientation of captured antibodies [21,22], with additional advantages of easy handling and high reaction kinetics [23,24]. Therefore, the combination of screen printed electrodes with MBs is presented as a valuable alternative for the development of electrochemical immunosensors to determine pathogenic substances [25].

The detection of microorganisms can be performed using their endogen enzymes such as β-galactosidase (β-gal), in order to obtain an unequivocal signal of their presence, avoiding the use of labeled antibodies. Methods that measure the activity of the β-gal endogenous enzyme were developed for the detection of coliform bacteria in water. In this way, chromogenic substrates are used such as o-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-β-D-galactopyranoside; or fluorogenic substrates such as 4-methylumbelliferyl-β-D-galactopyranoside [26]. Another alternative is the development of electrochemical immunosensors based on p-aminophenyl-β-D-galactopyranoside (p-APG) as the enzymatic substrate, which is hydrolized by β-gal to p-aminophenol (p-AP), which is electro-oxidized on the electrode surface at a potential close to 0.2 V [27–30]. In addition, square wave voltammetry (SWV) is a very good technique for electroanalytical purposes. It incorporates the best features of several voltammetric techniques, and has been established as a very reliable analytical technique widely recognized as one of the most sensitive electrochemical methods due to its ability to discriminate against the capacitive currents [31].

In this work, we discuss the development of an electrochemical magneto immunosensor (EMI) to detect and quantify ETEC F4 in swine feces through a direct non-competitive immunoassay. The EMI was based on MBs conjugated with polyclonal antibodies anti ETEC F4 (E-pAb) on carbon screen printed electrodes (CSPE). Once formed the immunocomplex on the electrode surface (ETEC F4/E-pAb/Mbs/CSPE), the β-gal endogenous enzyme in the presence of p-APG produces p-AP, which is oxidized on the CSPE using SWV. The EMI can detect and quantify ETEC F4 in swine feces samples at a very low concentration. A calibration curve was performed in the concentration range from 10^1 to 10^7 CFU mL^{-1}. Results obtained with the EMI are in very good agreement with those obtained by the conventional method of culture and plate count.

2. Materials and methods

2.1. Chemical and biological reagents

MBs (Dinabeads®, Invitrogen) derivatized with Protein G were used for a convenient orientation of the capture antibody. The MBs (2.8 μm diameter) have a high binding capacity, approximately 8 μg human IgG per mg of MBs. Before use, the MBs were loaded with saturating amounts of E-pAb as described below. Sheep polyclonal antibody anti *E. coli* K88ab (E-pAb) (2.7 mg mL^{-1}) was Abcam. p-APG, p-AP and Isopropy-β-D-thiogalactopyranoside (IPTG) were from Santa Cruz Biotechnology, Inc. Polymyxin B sulphate (PMBS) was Fluka Biochemika. Buffer solutions were prepared from their salts (Merck, p.a.): 1×10^{-2} M KH_2PO_4 + Na_2HPO_4, 0.14 M NaCl and 2.7×10^{-3} M KCl, pH 7.0 (PBS); and 5×10^{-2} M KH_2PO_4 + Na_2HPO_4, pH 6. PBS was used to prepare the E-pAb, the swine feces samples, and bacterial suspensions. The pH 6 phosphate buffer solution was used to prepare p-APG, p-AP, and PMBS solutions.

Samples of swine feces were given away by the Group of Salud Porcina, Departamento de Patología, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto. Samples were taken from the rectum of healthy adult animals, i.e., ranging in age from 4 to 22 weeks. They were obtained from confined farms in the towns of Salsipuedes and Baigorria, Province of Córdoba, Argentina.

2.2. Equipment and electrodes

Electrochemical measurements were performed with a potentiostat PGSTAT 101 Autolab (EcoChemie, Utrecht, The Netherlands), controlled by the NOVA 1.7 software. The CSPE based on working and counter electrodes of carbon and a pseudo-reference electrode of silver were purchased to Palm Sens (The Netherlands). The CSPE surface was electrochemically pre-treated before using it in 30 μL of 0.10 M KOH aqueous solution applying a potential step of 1.2 V for 5 min, following a procedure previously described by Anjo et al. [32]. Density optical (DO) measurements were performed using a Spectrum SP 2000 UV–visible spectrophotometer.

2.3. Bacterial strain

The reference strain of swine enterotoxigenic *E. coli* was given away by the Group of Biotecnología Animal, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto. It corresponds to a regional isolation and was characterized in the *E. coli* Reference Laboratory (Lugo-Spain) [33]. The strain has the protein aceous fimbrial antigens F4 (K88+) and F18, as determined by PCR analysis. It is a producer of heat-labile Toxin (LT) and heat-stable Toxin b (STb).

2.4. Culture medium and ETEC F4 suspensions

The culture medium used in all experiments was trypticase soy (Britania™) previously autoclaved during 15 min at 121 °C. ETEC F4 suspensions were prepared by inoculating 5 mL of trypticase soy broth with an isolated colony during 18 h at 37 °C. The concentration of overnight suspension was estimated from OD measurements carried out at 625 nm using the Mc Farland method. Therefore, ETEC F4 suspensions at different concentrations were prepared in PBS, and the OD of each suspension was measured. Simultaneously, each ETEC F4 concentration was determined the conventional method of culture and plate count. From OD values of each suspension *vs* concentration of ETEC F4 ($c^*_{ETEF\,F4}$), expressed as CFU mL^{-1} a good linear correlation was obtained. It come be expressed using the least square procedure as:

$$OD = (4.7 \pm 2.9) \times 10^{-2} + (6.2 \pm 0.4) \times 10^{-10} \times c^*_{ETEF\,F4} \quad r = 0.9874 \tag{1}$$

A 1:10 dilution in trypticase soy broth was performed with 5 mM IPTG for the EMI measurements. This dilution was incubated during 30 min at 37 °C for inducing the β-gal production. Ten-fold serial dilutions in PBS were made from this dilution before measurements.

2.5. Preparation of swine feces samples

1 g of swine feces was added to 10 mL of PBS. The suspension was stirred and then centrifuged during 10 min at 500 rpm. The super-

natant was separated in two fractions of 4.5 mL. One of them was inactivated by autoclaving during 40 min at 121 °C, and the another was stored at 4 °C until use. Before measurements, 500 µL of an ETEC F4 suspension, approximately 1×10^4 CFU mL^{-1}, was added to each fraction. This spiked swine feces samples were used with EMI.

2.6. Electrochemical measurements by square wave voltammetry

The quantification of TECT F4 was performed by SWV. SWV parameters were a frequency (f) = 25 Hz, square wave amplitude (ΔE_{sw}) = 50 mV and staircase step height (ΔE_s) = 5 mV. An equilibration time of 20 s was used. The interval of potential was from −0.2 to 0.5 V. The electrochemical response of p-AP (1×10^{-4} M in PBS) was also studied by cyclic voltammetry (CV). The scan rate (v) was 0.100 V s^{-1}.

2.7. Optimization of variables to determine ETEC F4

All variables involved in the β-gal generation were optimized in order to obtain the highest amount of p-AP when the enzymatic substrate was added. Therefore, a suspension of ETEC F4 in trypticase soy broth + IPTG was incubated at 37 °C. Then, dilutions at a final concentration of 10^7 CFU mL^{-1} were performed in PBS, and PMBS was added. Suspensions were incubated at room temperature under stirring at 1500 rpm. Finally, 20 µL of permeabilized bacterial suspension was dropped on the electrode surface and 10 µL of 1 mg mL^{-1} p-APG solution was added. SWV measurements were performed after 30 min.

2.7.1. Optimization of IPTG concentration and the incubation time with IPTG

From the overnight culture, ETEC F4 suspensions were prepared in trypticase soy broth with different IPTG concentrations, which were in the range from 0.2 to 10 mM. Each suspension was incubated during 30 min at 37 °C. Dilutions were performed in PBS and, then PMBS was added and incubated during 20 min. Finally, 20 µL of each suspension were dropped on the electrode surface, and SWV measurements were performed after of 30 min.

On the other hand, once the optimal IPTG concentration was determinated, the incubation time of ETEC F4 suspension in presence of IPTG was varied from 30 to 180 min. PMBS was added to each dilution and incubated. The SWV measurements were performed as it was previously described.

2.7.2. Study of the permeabilization time and SPM concentration on current values

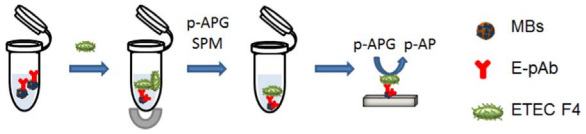
The permeabilization time (t_p) of ETEC F 4 was varied in the range from 5 to 90 min using a 10 µg mL^{-1} PMBS. When the optimal t_p was reached, the PMBS concentration was optimized. It was varied from 0.1 to 100 µg mL^{-1}. SWV measurements were performed for both experiments.

2.7.3. Optimization of the reaction time between p-APG and β-gal endogenous enzyme and the p-APG concentration

Once the β-gal production was optimized, a solution of p-APG was added to the ETECT F4 suspension. The enzymatic reaction generated produced p-AP. The time needed to produce p-AP is the reaction time (t_r). Thus, the t_r was studied in the range from 5 to 30 min. Then, the p-APG concentration was optimized at a t_r = 30 min (see below). Thus, the p-APG concentration was varied from 0.01 to 2 mg mL^{-1}. For each t_r and p-APG concentration, SWV measurements were performed.

2.7.4. Optimization of MBs volume on the electrochemical responses

The magnitude of SWV net peak currents ($I_{p,n}$) was evaluated for different MBs volumes which were between 0.5 and 2.5 µL. Thus, MBs were added to the ETECT F4 suspension. After the incubation and washing steps, the ETEC F4/E-pAb/MBs complexes were re-suspended in a pH 6 phosphate buffer solution, and a p-APG + PMBS solution was added. SWV measurements were performed after 30 min.



Scheme 1. Schematic representation of the electrochemical magneto-immunosensor.

2.7.5. Optimization of E-pAb concentration

2 µL of MBs were added to 50 µL of E-pAb solutions in the concentration range from 1 to 10 µL mL^{-1}. The solutions were incubated during 1 h at room temperature, stirring at 1500 rpm. Then, three consecutive steps of magneto-precipitation and washing were performed. Then, an ETEC F4 dilution was added and allowed reacting during 30 min. Steps of magneto-precipitation and washing were performed, and 50 µL of a 2 mg mL^{-1} p-APG + 10 µg mL^{-1} PMBS solution was added. Finally, SWV measurements were carried out after 30 min.

2.8. Electrochemical immunoassays to determine ETEC F4

A heterogeneous direct non-competitive immunoassay was used to detect ETEC F4 using the EMI (Scheme 1). Briefly, suspensions of 1 µL of MBs were transferred to Eppendorf tubes and washed three times with PBS to remove the NaN_3 used as preservative. Then, 50 µL of an E-pAb solution was added and stirred at 1500 rpm at room temperature during 1 h to obtain E-pAb/MBs complexes. After incubation, a high magnetic field was used for separating the MBs loaded with the antibody. The E-pAb/MBs complexes were washed with PBS and re-suspended in 50 µL of PBS. Then, this suspension was added to 1 mL of the ETEC F4 dilution, prepared from culture of a trypticase soy broth + IPTG, and incubated during 30 min at room temperature stirring at 1500 rpm. Thus, an ETEC F4/E-pAb/MBs complex was obtained.

The immuno-magnetic complex was washed and re-suspended in 20 µL of PBS. Then it was transferred to CSPE using a micropipette. Then, 10 µL of a solution PMBS + p-APG was added. SWV measurements were performed after 30 min. The $I_{n,p}$ were proportional to ETEC F4 concentrations.

2.9. Comparison of performance of EMI with the conventional microbiological techniques

The performance of EMI was evaluated respect to a reference method. Thus, the concentration value obtained using the EMI was compared with that obtained with the conventional method of culture and plate count used as the reference method. Thus, ETEC F4 suspensions between 10^2 and 10^3 CFU mL^{-1} were prepared in pH 6 phosphate buffer solution, and were inoculated on trypticase soy agar in Petri dishes. Enumeration of colonies was performed after 24 h of incubation at 37 °C. On the other hand, concentrations of the same ETEC F4 suspensions were determined from the corresponding calibration curve obtained with EMI.

3. Results and discussion

3.1. Electrochemical oxidation of p-AP

It is known that the p-AP oxidation corresponds to a quasi-reversible redox couple [34]. Thus, the electrochemical oxidation of p-AP in PBS was performed using CV and SWV (Fig. 1). An oxidation peak was observed by CV, with a peak potential centred at 0.15 V (Fig. 1a). The complementary cathodic peak centred at 0.10 V was found when the potential sweep direction was reversed. A difference between anodic and cathodic potential peaks (ΔE_p) of 65 mV was

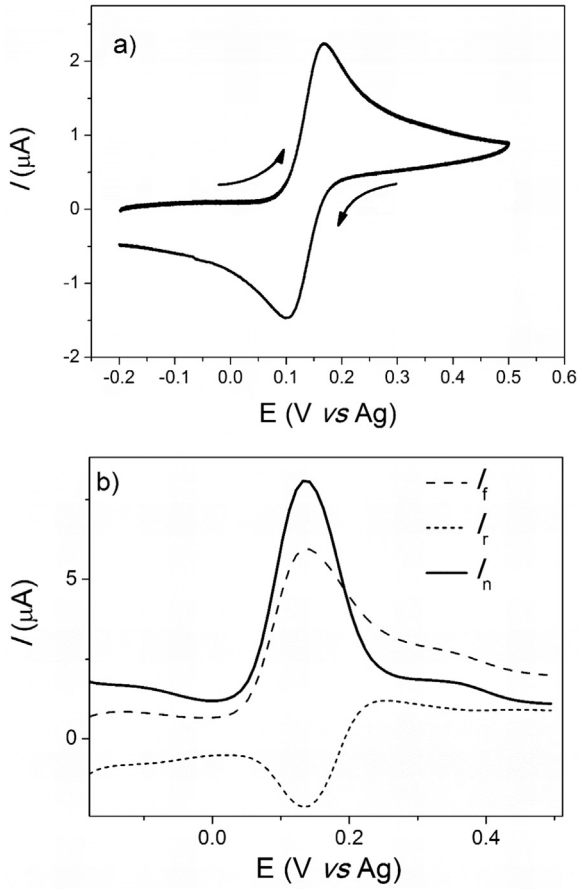


Fig. 1. Cyclic (a) and square wave (b) voltammograms obtained using a CSPE. c^*_{p-AP} = 1×10^{-4} M in PBS. CV parameter: v = 0.100 V s^{-1}. SWV parameters: ΔE_{sw} = 50 mV, ΔE_s = 5 mV and f = 25 Hz. I_f: forward, I_r: reverse and I_n net currents, respectively.

obtained. On the other hand, the net current (I_n) of SW voltammograms recorded for p-AP shows a peak centred at 0.135 V (Fig. 1b), with contribution of the forward (I_f) and reverse (I_r) currents as it is expected for a quasi-reversible redox couple [31]. On the other hand, p-APG was also studied by CV and SWV. In both cases, any peak system was found. Then, p-AP produced enzymatically was used to determine ETEC F4 using the EMI.

3.2. Optimization of the IPTG concentration and the incubation time of ETEC F4 with IPTG

IPTG induces the production of β-gal endogenous enzyme. Thus, the $I_{n,p}$ was studied for different concentrations of IPTG in a ETEC F4 suspension in trypticase soy broth. The $I_{n,p}$ increases as the IPTG concentration was increased, reached a maximum value for c^*_{IPTG} = 5 mM (Fig. 2). The incubation time of ETEC F4 in a of trypticase soy broth with a c^*_{IPTG} = 0.5 mM was also studied. The inset of Fig. 2 shows the dependence between $I_{n,p}$ with the incubation time. A constant values of $I_{n,p}$ was obtained in the time range from 30 to 180 min, indicating that the production of β-gal is independent of time for a c^*_{IPTG} = 0.5 mM. Thus, a c^*_{IPTG} = 5 mM and an incubation time of 30 min were chosen for further experiments.

3.3. Results obtained for the optimization of the permeabilization time and SPM concentration

The permeabilization of cellular wall was studied through the variation of $I_{n,p}$ with t_p (see Section 2.7.2). Fig. 3a shows an increase of $I_{n,p}$ up to a t_p ≈30 min. For $t_p \geq 40$ min, the $I_{n,p}$ tends to reach a

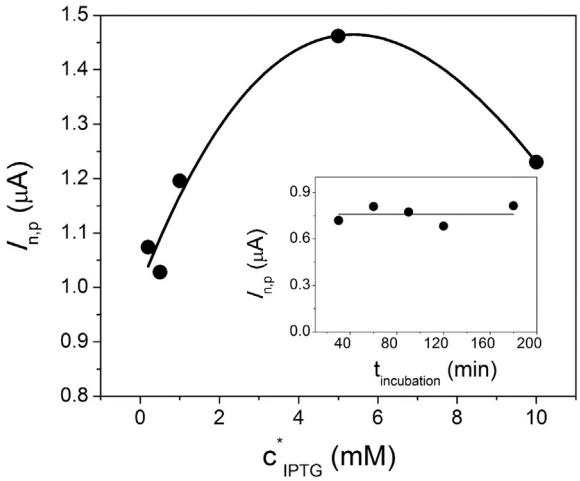


Fig. 2. Dependence of $I_{n,p}$ as a function of c^*_{IPTG}.The inset shows the dependence of $I_{n,p}$ with ETEC F4 incubation time in solutions of c^*_{IPTG} = 0.5 mM. $c^*_{ETEC\ F4}$ = 1.60×10^7 CFU mL^{-1}.

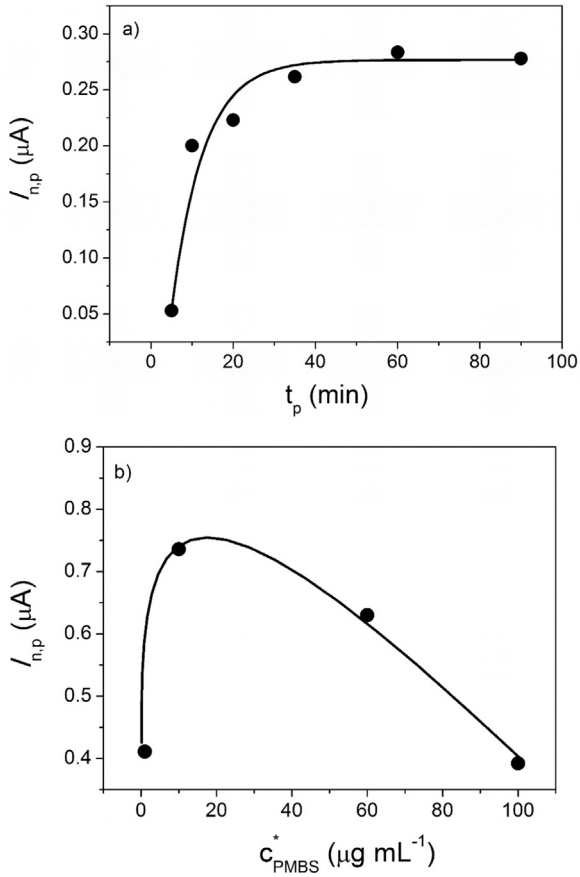


Fig. 3. a) Dependence of $I_{n,p}$ with the permeabilization time (t_p). c^*_{PMBS} = 10 μg mL^{-1}. b) Variation of $I_{n,p}$ for different PMBS concentrations. t_p = 30 min $c^*_{ETEC\ F4}$ = 1.50×10^7 CFU mL^{-1}.

stationary value. On the other hand, the effect of PMBS concentration in $I_{n,p}$ was also studied. The t_p was of 30 min. This t_p guarantees an optimal permeabilization of ETEC F4. The $I_{n,p}$ increases as the c^*_{PMBS} increases, and a maximum value is reached for c^*_{PMBS} = 10 μL mL^{-1}. For $c^*_{PMBS} \geq$ 10 μL mL^{-1}, the $I_{n,p}$ decreases (Fig. 3b). Thus, a t_p = 30 min and c^*_{PMBS} = 10 μL mL^{-1} were chosen for all next experiments.

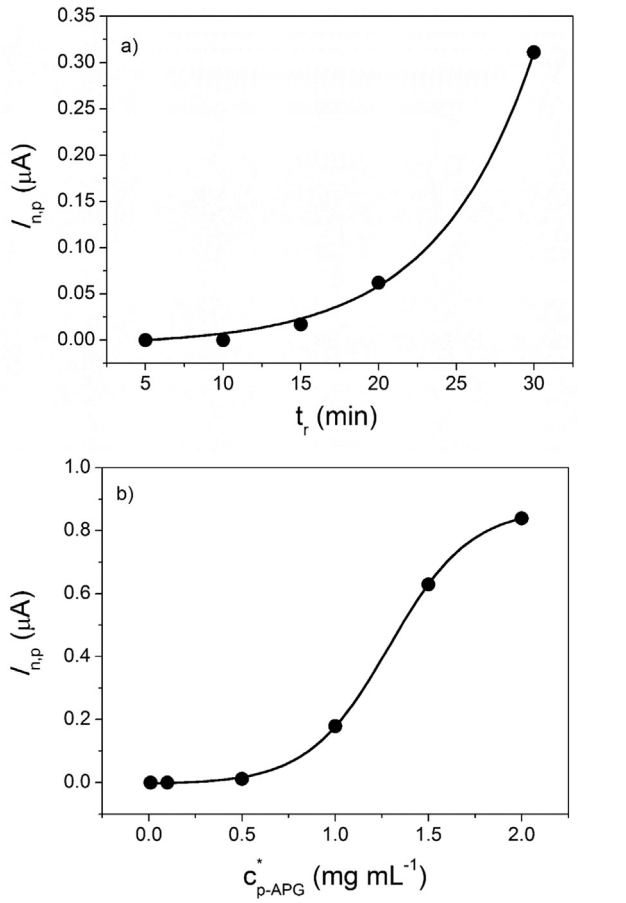


Fig. 4. a) Dependence of $I_{n,p}$ with the reaction time (t_r) between p-APG and β-gal. b) $I_{n,p}$ as a function of c^*_{p-APG}. $c^*_{ETEC\ F4}$ = 1.70×10^7 CFU mL^{-1}.

3.4. Optimization of the reaction time between p-APG and β-gal endogenous enzyme and of p-APG concentration on the electrochemical responses

When the t_r between the β-gal endogenous enzyme of ETEC F4 and p-APG increases, the $I_{n,p}$ grows exponentially (Fig. 4a). Thus, as the t_r increases, the production of p-AP by the reaction between β-gal and p-APG is also increased. However, a minimum time of 10 min is necessary to have a significant difference in the values of $I_{n,p}$. A t_r = 30 min was chosen for all experiments. In addition, $t_r \geq 30$ min were discarded to avoid an unnecessary increase in the time required for the ETEC F4 detection. On the other hand, an increase in p-APG concentration produces a continuous increase of $I_{n,p}$, indicating a higher production of p-AP (Fig. 4b). A c^*_{p-APG} = 2 mg mL^{-1} was chosen for the ETEC F4 detection. For $c^*_{p-APG} \geq 2$ mg mL^{-1}, p-APG starts to hydrolyze and p-AP appears in the absence of β-gal (data not shown).

3.5. Optimization of the MBs volume

The optimization of the volume of MBs suspensions was performed in the conditions previously determined. Fig. 5 shows that the $I_{n,p}$ reached a maximum value when the volume of MBs was 1 μL. For a higher MBs volume, the immunocomplex ETEC F4/Ep-Ab/MBs deposited on CSPE partially prevents the oxidation of p-AP on the electrode. Thus, a volume of MBs of 1 μL was chosen.

3.6. Optimization of the E-pAb concentration

The ETEC F4/E-pAb/MBs complex was generated for the E-pAb concentration range described in Section 2.7.5. $I_{n,p}$ values increase as

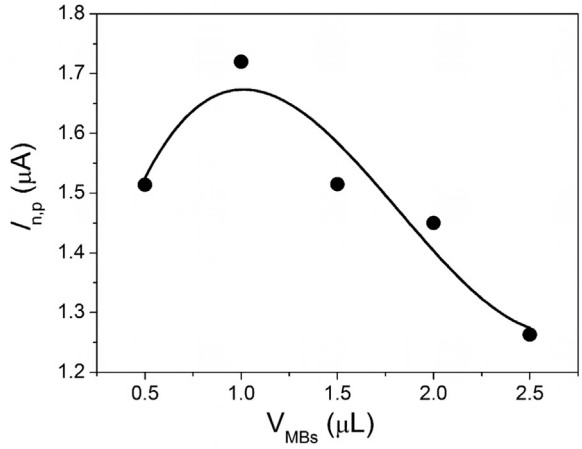


Fig. 5. Dependence of $I_{n,p}$ with the volume of MBs (V_{MBs}). $c^*_{ETEC\ F4}$ = 1.60×10^5 CFU mL^{-1} and c^*_{p-APG} = 2 mg mL^{-1}.

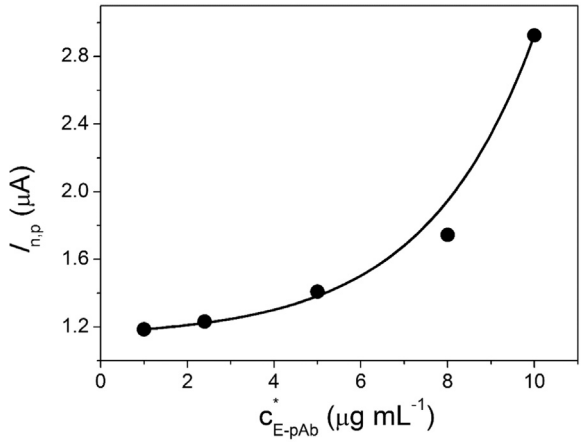


Fig. 6. Dependence of $I_{n,p}$ with the E-pAb concentration.$c^*_{ETEC\ F4}$ = 1.80×10^5 CFU mL^{-1}.

the c^*_{E-pAb} was increased (Fig. 6). A c^*_{E-pAb} = 10 μg mL^{-1} was used to develop the EMI. A $c^*_{E-pAb} \geq 10$ μ g mL^{-1} were not used to avoid unstable agglomerations of E-pAb.

3.7. Calibration curve for ETEC F4

A calibration curve for ETEC F4 was carried out in the concentration range from 10^1 to 10^7 CFU mL^{-1} (Fig. 7) using the EMI. Each point is an average of five replicated measurements in five different days. The calibration curve was plotted as the relationship between $I_{n,p}$ and $I^0_{n,p}$, where $I^0_{n,p}$ is the current obtained when the ETEC F4 saturation is reached. The inset of Fig. 7 shows SW voltammograms obtained for each ETEC F4 concentration of calibration curve.

The linear range corresponds to ETEC F4 concentrations in the range from 5×10^1 to 5×10^3 CFU mL^{-1}, and the sensibility (slope of linear portion) was 4×10^2 CFU^{-1} mL. The limit of detection (LOD), calculated as the concentration of ETEC F4, which causes an increase in signal equal to three times the standard deviation of the blank [35], was 33 CFU mL^{-1}. The value of LOD is significantly lower with respect to those reported for the same analysis time (Table 1), i.e., LOD values higher than 10^3 CFU mL^{-1}. Thus, the EMI allows detecting ETEC F4 in incipient stage of swine disease [36].

3.8. ETEC F4 determination in swine feces

Feces of healthy swine were separated in two portions. Both portions were 1:10 diluted in PBS. One of them was inactivated in an autoclave. Then, both portions were spiked with 1.6×10^3 CFU mL^{-1}

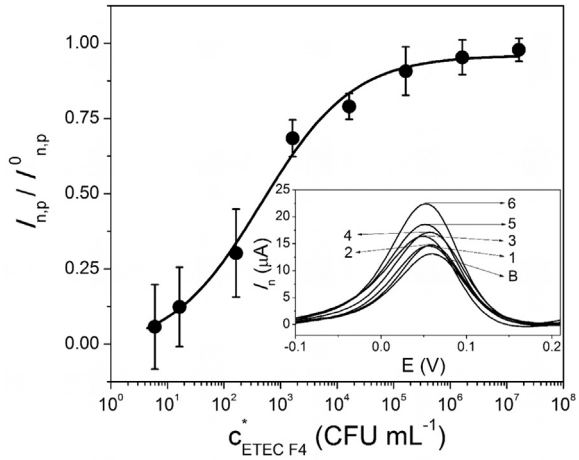


Fig. 7. Calibration curve obtained for ETEC F4 using the EMI. Each point is the average of five replicated measurements. The inset plot shows the square wave voltammograms recorded for different ETEC F4 concentrations: B) 0; 1) 10^1; 2) 10^2; 3) 10^3; 4) 10^4; 5) 10^5 and 6) 10^6 CFU mL^{-1}.

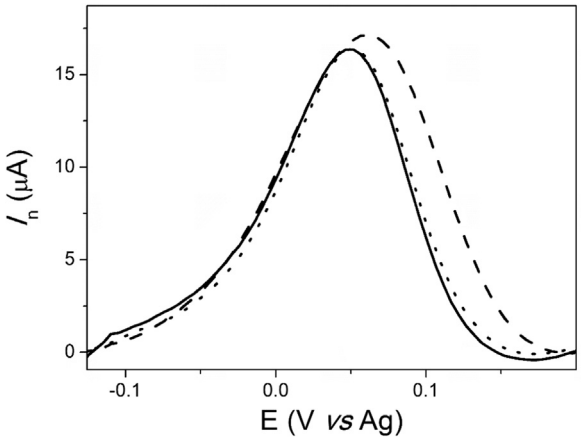


Fig. 8. Net currents of square wave voltammograms obtained for the 1.6×10^3 CFU mL^{-1} ETEC F4 suspensions: (...) PBS, (—) the swine feces suspension, and (—) the inactivated swine feces suspension.

ETEC F4. Fig. 8 shows the SW voltammograms obtained for the swine feces samples diluted in PBS, which one of them was inactivated and the another no, and a suspension of 1.6×10^3 CFU mL^{-1}of ETEC F4 in PBS. Before to perform the SW voltammograms, the three suspensions were centrifuged at 500 rpm. The $I_{n,p}$ obtained were close to each other, indicating a good selectivity of the EMI. The slightly higher $I_{n,p}$ of inactivated suspension was due a slight reduction of volume of the sample during the autoclaving.

3.9. Performance of EMI

The results obtained with the EMI were compared with the conventional method of culture and plate count [37–41]. Thus, an overnight culture of ETEC F4 was prepared from a ETEC F4 colony isolated from trypticase soy agar (incubating during 18 h at 37 °C). A concentration of 2.20×10^8 CFU mL^{-1} of ETEC F4 was determined by turbidimetry using the Mc Farland method, through optical density measurements at 625 nm. Ten-fold serial dilutions were performed on overnight culture using PBS to achieve an ETEC F4 concentration of 2.20×10^2 CFU mL^{-1}. 100 μL of this suspension was added to trypticase soy agar and it was incubated during 24 h at 37 °C. Then, a colonies count was performed. This experiment was performed by triplicate. The ETEC F4 concentration obtained was $(1.82 \pm 0.45)\times10^2$ CFU mL^{-1} (%E_r = 25%). For the same ETEC F4 suspension, the ETEC F4 concentration determined by the EMI through three replicated measurements was to $(4.1 \pm 0.8)\times10^2$ CFU mL^{-1}, (%E_r = 20%). The average of $I_{n,p}$ / $I^0_{n,p}$ obtained was (0.52 ± 0.10). This result shows the very good performance of EMI. Thus, the EMI appears as a sensible, fast and economic tool to determine ETEC F4 in swine feces samples.

4. Conclusions

In this work, an electrochemical magneto-immunosensor has been developed for a selective detection and quantification of ETEC F4 in swine feces samples using the β-gal endogenous enzyme to generate p-AP from p-APG. The p-AP was oxidized on CSPE. SWV, a very fast and sensitive electrochemical technique, was used to determine the p-AP, allowing reaching a very low limit of detection (33 CFU mL^{-1}). A calibration curve with a wide linear range was obtained (from 5×10^1 to 5×10^3 CFU mL^{-1}). All variables of the construction of EMI were optimized. The ETEC F4 suspensions were treated with IPTG, allowing a major production of β-gal, and with PMBS to obtain a better contact with p-APG. Thus, a lesser time of analysis and higher sensibility were reached.

The EMI has several advantages such as the unnecessary use of a second antibody labeled with an enzyme. The EMI can detect ETEC F4 in swine feces samples without any pre-treatment or bacterial enrichment assays. It is selective and the analysis time is short compared to the culture method and plate count. On the other hand, a very good agreement between results obtained with the EMI and those obtained with the culture method and plate count was found. Compared to other techniques of bacterial quantification, the EMI is one of the most

Table 1
Comparison of methods for *E. coli* determination based on the β-gal enzyme.

Method	LOD (CFU mL^{-1})	Assay Time	Matrix	Ref.
Amperometry	5×10^4	2 h	Luria Bertani Medium	[27]
	10	7 h	River water samples	
Chronocoulometry	10^5	1 h	Luria Bertani Medium	[29]
	1	4 h		
Amperometry	10	5 h	Luria Bertani Medium	[34]
	10^2	4 h	River water samples	
Amperometry	10^5	5 h	Luria Bertani Medium	[36]
	10^2	6 h		
	1	8 h		
Fluorescence and Chemiluminescence	1 CFU/100 mL	4 h	Water samples	[37]
Fluorescence	92	20 min	Wastewater treatment plants	[38]
Fluorescence	9.6×10^2	40 min	*E. coli* suspensions in 0.9% NaCl water	[39]
	1.5×10^3	~ 5 h	Wastewater	
Amperometry	10^6	Incubation during 3 h – time detection: 10 min	PBS	[40]
	10	Incubation during 5 h – time detection: 10 min		
SWV	33	2 h	PBS	This work

sensitive. Thus, we conclude that this EMI is a reliable tool to detect and quantify ETEC F4 in swine feces samples.

Acknowledgments

Financial support from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) under the Grant PIP 112-201101-00184, Agencia Nacional de Promoción Científica y Tecnológica (FONCYT) under the Grant PICT 0916/2010 and PICT 2266/2014, Ministerio de Ciencia y Tecnología de la Provincia de Córdoba (MinCyT) under the Grant PID 050/2010, and Secretaría de Ciencia y Técnica from the Universidad Nacional de Río Cuarto (UNRC) under the Grant Res. Rec. 1230/14 are gratefully acknowledged. We thank Professors C. Errecalde, G. Prieto and F. Mañas for allowing the use of their laboratories for electrochemical measurements with bacteria, and to Professors J. J. Busso, A. Carranza and J. Parada for providing the stool samples. Also to Lic. C. Basualdo by her valuable help in laboratory tasks at the Facultad de Agronomía y Veterinaria, UNRC. L. V. Tarditto thanks to CONICET for a doctoral fellowship.

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