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Electrochemical sensor for the determination of enterotoxigenic *Escherichia coli* in swine feces using glassy carbon electrodes modified with multi-walled carbon nanotubes

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ABSTRACT

Enterotoxigenic *Escherichia coli F4* (K88) (ETEC *F4*) is one of the main etiologic agents involved in neonatal and post-weaning diarrhea in pigs, which generates significant economic losses due to high mortality and morbidity. An electrochemical sensor based on glassy carbon electrodes modified with a dispersion of multi-walled carbon nanotubes plus 1% Nafion™ is proposed to detect and quantify ETEC *F4* in stool samples of pigs. The preparation of samples was made by dilution in phosphate buffer solutions (PBSs), followed by centrifugation and inactivation of the supernatant in autoclave. An irreversible oxidation peak was observed by cyclic voltammetry at a potential close to 0.69 V. The best response was obtained for an optimum pre-concentration time of 10 min under forced convection conditions. The electrochemical response performed by square wave voltammetry was obtained in 1/5000 fecal samples in PBS spiked with ETEC *F4*. A peak current vs. ETEC *F4* concentration plot was constructed from successive additions of suspensions of ETEC *F4* inactivated by autoclave. The limit of detection (LOD), the limit of quantitation (LOQ) and the relative standard deviation were 6×10^4 CFU mL^{-1}, 2×10^5 CFU mL^{-1} and 20%, respectively. The developed electrochemical method is simple, fast and economical to quantify ETEC *F4* in swine stool samples, making it useful for diagnosis monitoring in production facilities.

1. Introduction

Diarrheal infections or colibacilosis in pigs generate significant economic losses due to high mortality rates, decreased weight gain, delay in the production and/or reproductive performance and increased expenses due to the actions taken against the presence of a disease (treatment, vaccinations, sacrifices, diagnostic tests, etc.) [1]. One of the main etiologic agents involved in neonatal and post-weaning diarrhea (PWD) is enterotoxigenic *Escherichia coli F4* (K88) (ETEC *F4*) bacteria [2,3]. Moredo et al. indicate that for 95.5% of PWD, ETEC *F4* is the triggering agent [3]. The bacterium ETEC *F4* constantly spreads in the immediate surroundings of the pig, mainly through feces, food or water contaminated with feces and infection occurs by the fecal-oral [4].

The fimbriae are one of the virulence factors of ETEC *F4*. They are long proteinaceous appendages radiating from the surface of the bacterium with a length of about 0.5 ± 1.5 μm. Fimbriae are spread peritrichously on bacteria [5]. In the structure of these protein appendages are *F4* and F18 antigens, with which the bacterium adheres to the microvilli of enterocytes early in infection.

A presumptive diagnosis of bacterial infections based on the clinical picture, the risk factors associated with the pigs themselves and the environment where they are, is usually done in veterinary practice [6].

To avoid the use of broad-spectrum antibiotics and the generation of multiple resistances, it would be desirable to have a simple diagnostic tool that quickly allows monitoring the sanitary status of the production facilities.

The detection of pathogenic microorganisms is carried out by techniques based on typing and culture, molecular techniques (mainly polymerase chain reaction, PCR) and immunological methods (ELISA). The first, less expensive, may have limitations for routine use due to the time required to obtain results with trained professionals [7]. It is desirable to have other rapid and sensitive tools that help monitor the presence of pathogens in minimally equipped laboratories and/or in field applications. In this regard, numerous investigations are underway aimed to develop sensors for the detection of bacteria for use primarily in the food industry because of the impact on human health [8–10].

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Thus, several methods for rapid detection of *E. coli* O157:H7 have been developed. They are based on PCR [11,12], immunoassays with enzymes and various platforms (ELISA) [13], immunomagnetic methods [14] or microchips-PCR arrays [15] and also several types of biosensors [16–22], including aptamers with colorimetric detection [23].

Other devices are based on nanotechnology tools and electrochemical transducers [24,25]. In these cases, electrochemical techniques routinely employed are impedance spectroscopy, cyclic voltammetry (CV), chronoamperometry and square wave voltammetry (SWV), sometimes combined with chemometric tools [26].

SWV has great advantages over other electrochemical techniques, including increased sensitivity, short analysis time and a significant decrease of capacitive currents [27]. It has also been used to quantify bacterial cultures [26,28].

The use of modified electrodes allows improving the sensitivity, selectivity, reproducibility and/or stability of electrochemical sensors. Multi-walled carbon nanotubes (MWCNTs) have been used in the development of sensors mainly because of their high electrical conductivity and high surface/volume ratio [29,30]. Their catalytic and amplification signal effects have been demonstrated in the electrochemical determination of biological molecules such as cytochromes [31], NADH [32] and bacteria [28], among others.

The objective of this work is to develop an electroanalytical sensor with SWV detection to monitor ETEC *F*4 bacterial populations directly in stool samples of pigs. The sensor consists of a glassy carbon disk electrode (GCE) modified with a dispersion of MWCNT in 1% Nafion™ aqueous solution. The electrochemical responses of suspensions of live bacteria as well as bacteria inactivated in autoclave in phosphate buffer solutions (PBS) were analyzed. The quantification was performed in a concentration range between 6×10^6 and 3×10^7 CFU mL^{-1} (CFU = live bacteria colony forming units). The electrochemical sensor performance was checked on stool samples of pigs inoculated with ETEC *F*4 bacteria. As far as we know, this is the first time that the approach proposed is used in this complex matrix.

2. Material and methods

2.1. Chemicals

MWCNTs (110–170 nm dia., 8–9 μm in length) were obtained from Sigma. They were oxidized in (2:1) H_2SO_4/HNO_3 by heating under reflux during 10 h to facilitate their dispersion in water. Then, they were purified by centrifugation at 4000 rpm, the supernatant was removed and they were washed with distilled water. This procedure was repeated 3 times. Finally, the suspension obtained was dialyzed using a dialysis membrane of 100 kDa against distilled water until the pH of the water was close to 7. A pH 7 10 mM PBS was prepared from the corresponding phosphate salts. PBS was used to prepare bacterial suspensions, to dilute samples of swine feces and as the supporting electrolyte for electrochemical measurements. The 5% (w/w) perfluorinated Nafion™ was obtained from Aldrich.

2.2. Equipment and electrodes

Electrochemical measurements were performed with a potentiostat PGSTAT 101 Autolab (EcoChemie, Utrecht, The Netherlands), controlled by NOVA 1.7 software. A conventional glass cell of three electrodes was used. The working electrode was a glassy carbon disk (3 mm dia.) from BAS (USA) modified with MWCNT, the reference electrode was Ag/AgCl and the counter electrode was a Pt wire of large area ($A \approx 2$ cm^2). Measurements of optical density (OD) were performed with a Spectrum SP 2000 UV–Vis spectrophotometer. Phenomenex™ nylon filters were 25 mm dia. with 0.45 μm pores. They were autoclaved during 15 min at 121 °C.

2.3. Bacterial strain

The reference strain of swine enterotoxigenic *E. coli* was given 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]. It was determined by PCR analysis that the strain has the proteinaceous fimbrial antigens *F*4 (K88+) and F18 and it is a producer of STb and LT toxins which disrupt intestinal fluid homeostasis to cause electrolyte-rich fluid hyper-secretion and diarrhea [34]. These toxins are the main responsible of swine diarrhea.

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. Suspensions of bacteria were prepared by inoculating 5 mL of trypticase soy broth with an isolated colony on trypticase soy agar and incubating at 37 °C at different incubation times (t_{inc}). After incubation, the culture was washed 3 times by centrifugation during 15 min at 2500 rpm, the supernatant was discarded and the sediment containing the bacteria was *re*-suspended in PBS. Thus, all components of trypticase soy broth are eliminated and are not oxidized in ETEC *F*4 determination. From this suspension, successive 1/10 dilutions in PBS were conducted for all electrochemical experiments.

The concentration of each suspension was estimated from the OD at 625 nm, following the well known Mc Farland method. Thus, suspensions of ETEC *F*4 in PBS at different concentrations were prepared, and the OD of each suspension was measured. Simultaneously, each concentration was determined by plate count. From OD values of each suspension vs. concentration (10^{-8} $c_{ETEC\ F4}$) expressed as CFU mL^{-1} a good linear correlation was obtained. It was expressed by the following equation:

$$OD = (4.7 \pm 2.9) \times 10^{-2} + (6.2 \pm 0.4) \times 10^{-2} * c_{ETEC\ F4} \quad r = 0.9874.$$

2.5. Preparation of samples

Samples of live and inactivated bacteria were prepared in PBS. Inactivated bacteria samples were used for measurements using the method of standard additions in stool samples of pigs. They were spiked with inactivated bacteria, which were given by the group of Salud Porcina from the Departamento de Patología, Facultad de Agronomía y Veterinaria, Universidad Nacional de Río Cuarto. The stool samples were taken from the rectum of healthy adult animals, i.e., ranging in age from 4 to 22 weeks, from confined farms in the towns of Salsipuedes and Baigorria, Province of Córdoba, Argentina. Transportation to the laboratory was performed using sterile containers refrigerated at 4 °C and stored at this temperature until use.

A suspension with 1 g feces in 5 mL of PBS was prepared and centrifuged during 10 min at 500 rpm. The electrochemical measurements were performed in the supernatant inactivated in autoclave and diluted to 1/5000 with PBS. We have found that the matrix effects are minimal under this condition. Successive additions of suspension of inactivated ETEC *F*4 of a given concentration in PBS were done, and a standard addition plot was obtained.

2.6. Working electrode modification

The GCE was pretreated through a mechanical polishing with 0.05 μm alumina on a damp cloth and then twice sonicated in distilled water at intervals of 1 min to remove residual alumina. It was rinsed with distilled water and dried in an oven at 37 °C. Then, the MWCNT dispersion was prepared by adding 1 mg of MWCNT to a 1% Nafion aqueous solution contained in an Eppendorf tube. The tube was then introduced in an ultrasonic bath during 30 min. Thus, a homogeneous dispersion was obtained. The modification of the GCE was performed

by placing 20 μL of 1 mg mL^{-1} MWCNT dispersion on the pretreated electrode and dried in an oven at 37 °C during approximately 30 min. Stabilizing the surface of the electrode was then carried out in PBS by performing 20 consecutive voltammetric cycles at 0.100 V s^{-1} in the −0.2 to 1 V potential window.

2.7. Electrochemical measurements

The electrochemical response of ETEC *F4* suspensions was analyzed by CV and SWV at GCE modified with MWCNT. The potential window for SWV was from 0.4 to 0.85 V. SWV parameters were: ΔE_{SW} = 25 mV, ΔE_S = 5 mV, f = 10 Hz and t_{eq} = 60 s, where ΔE_{SW} is the square wave amplitude, ΔE_S is the staircase height, f is the frequency and t_{eq} is the equilibration time.

3. Results and discussion

3.1. Electrochemical behavior of suspensions of live bacteria

As far as we know, no report on electrochemical behavior for ETEC *F4* is available in the literature. Cyclic voltammograms of suspensions of bacteria, prepared from a culture after 6 h of incubation, were obtained in a potential window from 0 to 1 V. As shown in Fig. 1, an irreversible oxidation peak appears at about 0.69 V, whose current intensity decreases after few consecutive scans. On the other hand, its peak potential (E_p) shifts slightly to more positive values. This behavior suggests the gradual passivation of the electrode surface, due to the possible blocking effect of electro inactive anodic reaction product/s.

The electrochemical response of suspensions of ETEC *F4* was also analyzed by SWV. Experimental net square wave currents (I_n) were corrected by subtracting blank currents. Thus, a sigmoid shape curve between the net peak current ($I_{p,n}$) and frequency (f) was obtained.

These results indicate that adsorption of the analyte (bacteria or their metabolites) would be coupled to charge transfer in the reaction mechanism [35]. This phenomenon is of great importance for the quantification of *E. coli* by pre-concentration on the modified electrode. Pre-concentration experiments were performed in stirred suspensions. The variation of $I_{p,n}$ vs. the pre-concentration time (t_{pre}) was analyzed. As shown in Fig. 2, net currents tend to reach a steady state after 10 min. Therefore, this time was selected as the optimum for the preconcentration.

The electrochemical behavior of certain living cells has been studied by several authors, who have suggested some mechanisms by which direct electron transfer occurs from microorganisms to the electrode

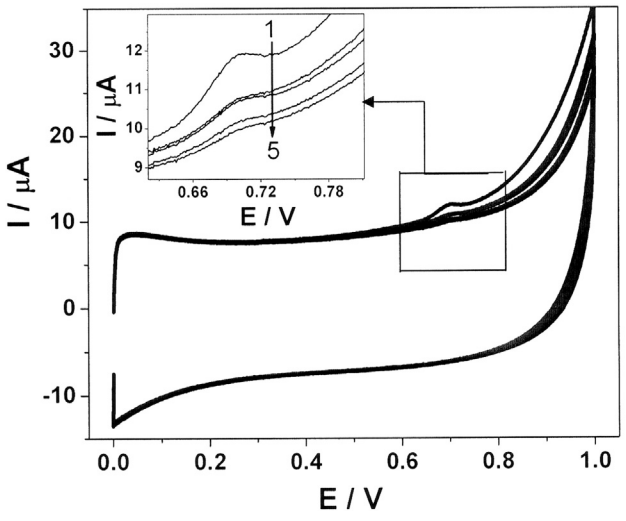

Fig. 1. Cyclic voltammograms of 2.0×10^8 CFU mL^{-1} ETEC *F4* suspensions in PBS. Working electrode: GCE-MWCNT. v = 0.050 V s^{-1}.

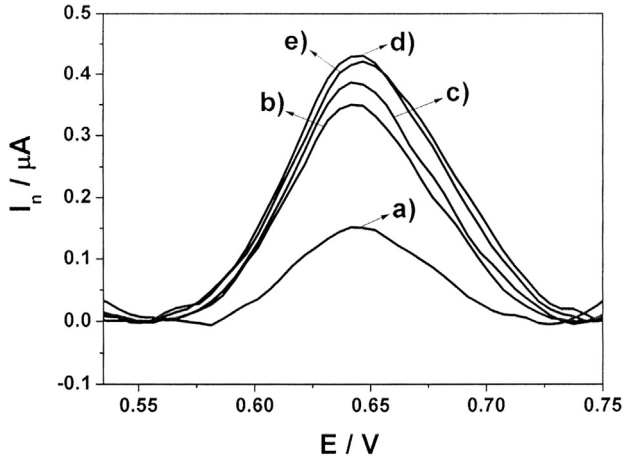

Fig. 2. Net currents of square wave voltammograms of a suspension of ETEC *F4* at different preconcentration times: a) 0; b) 2 min; c) 4 min; d) 8 min and e) 16 min. $c_{ETEC\ F4} = 1.7 \times 10^8$ CFU mL^{-1}.

[36–38]. These mechanisms include the production of soluble redox molecules which are released into the medium, direct contact between the outer membrane of the cells and the electrode [39] and the participation of the electron transport chain, related to the microbial respiration [40]. Despite all these suggestions, the mechanism of electron transfer between microorganisms and the electrode surface is not yet fully elucidated [41]. In fact, there are different molecules and/or macromolecules as possible candidates to be oxidized in the 0.55–0.75 V potential range in a suspension of ETEC *F4*, such as cytochrome *c*, ubiquinones and menaquinones, NADH, and succinate, which are both in the cell membrane and in the cytoplasm. They are capable of being oxidized in the potential range mentioned above. For this reason we have studied the response under both aerobic and anaerobic conditions, in the presence and in the absence of bacteria (prefiltration) and also with live or dead bacteria by cell lysis. In all cases we find an oxidation current of variable intensity in the same region of potential (see below).

In order to further investigate the possible origins of the electrochemical response of ETEC *F4* suspensions and optimize the conditions for bacteria detection, SWV experiments in the presence and in the absence of cells were performed. For this, a suspension of ETEC *F4* bacteria, *re*-suspended in PBS, was divided into two equal parts. Then, cells were filtered in one of them. In both cases it was observed that the oxidation occurs at approximately the same potential (Fig. 3).

From SW voltammograms of Fig. 3, it may be suggested that the electrochemical response would be related to the production of endogenous substances, which are released to the medium. In the presence of bacteria, the response could be enhanced due to the direct transfer between the microorganisms in contact with the electrode surface.

This suggests that $I_{p,n}$ would depend on the growth phase of the microorganisms (Fig. 4) due to the changes occurring in the permeability of cell membranes. Therefore, the electrochemical response of suspensions of bacteria prepared from inocula in the exponential and stationary phases was analyzed.

Net currents from exponential phase are close to three times higher than the net currents of stationary phase, due to an increased permeability of cell membranes. Then, a growth curve was performed by SWV and a similar behavior was found (data not shown).

The same tendency was observed with respect to the growth phase when the response of the filtrates of bacteria suspensions was analyzed, but signals have slightly lower current intensities.

Experiments were also performed in oxygen or argon atmosphere, with no significant differences between the square wave voltammograms. Therefore, it is probable that the electrochemical response of ETEC *F4* is due to the production of endogenous redox molecules and to the direct contact between the outer cell membrane and the

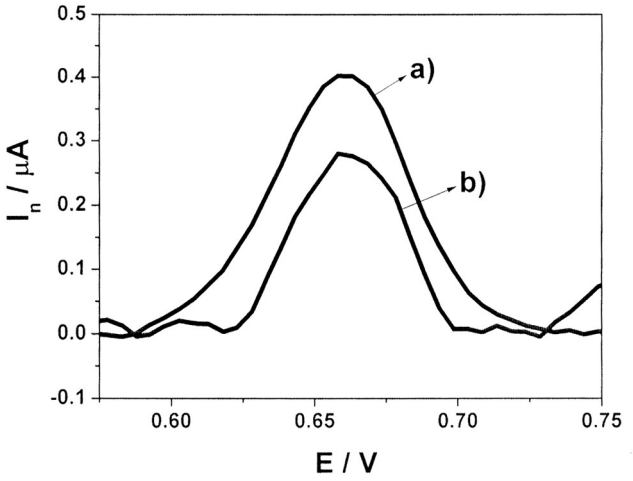

Fig. 3. Net currents of square wave voltammograms of a) 2.14×10^8 CFU mL^{-1} ETEC *F4* suspension, b) in the absence of bacteria.

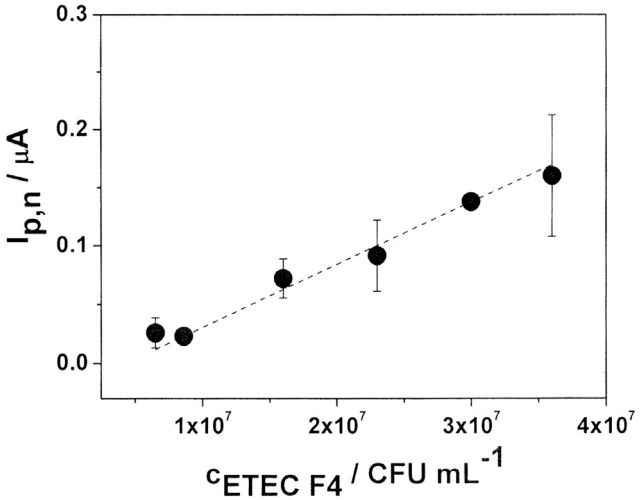

Fig. 5. Calibration curve of suspensions of live ETEC *F4* bacteria. Each point is an average of three experimental measurements. Intercept $= -(2.2 \pm 0.2) \times 10^{-2}$ μA; slope $= (5.3 \pm 0.1) \times 10^{-9}$ μA mL UFC^{-1}; r = 0.9990.

electrode surface in an aerobic and/or facultative aerobic respiration process. It has to be emphasized that this was not an impediment to quantify ETEC *F4*, since the magnitude of the oxidation current was, in fact, linearly proportional to the concentration.

3.2. Calibration curve for quantifying live bacterial suspensions

A linear relationship was found between $I_{p,n}$ and the concentration of bacteria in PBS (Fig. 5). The suspensions of bacteria were prepared from an exponential phase inoculum (t_{inc} = 6 h). The pre-concentration time was 10 min. From regression results, a sensitivity of $(5.3 \pm 0.1) \times 10^{-9}$ μA mL CFU^{-1} was obtained.

3.3. Inactivated bacteria suspensions. Calibration curve

Suspensions of bacteria were prepared as described in the experimental section. Their concentrations were determined by OD measurements (Section 2.4). Then, they were inactivated by autoclaving during 40 min at 121 °C. The electrochemical response of suspensions of inactivated ETEC *F4* (Fig. 6a) showed an E_p similar to suspensions of live bacteria, but with a higher $I_{p,n}$ and a slight shoulder before the main peak.

This result demonstrates that the electrochemical signal of ETEC *F4* would not be exclusive of viable cells, unlike other microorganisms [28,36,42,43]. Fig. 6b shows the calibration curve obtained in inactivated suspensions of ETEC *F4*. The variation of $I_{p,n}$ with $c_{ETEC\ F4}$ is typical of adsorbed reactant systems, where saturation is reached for high concentration of the analyte [44].

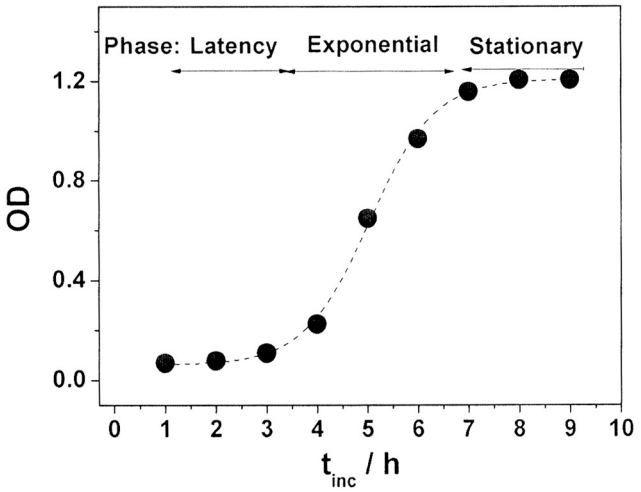

Fig. 4. ETEC *F4* growth curve. t_{inc}: incubation time.

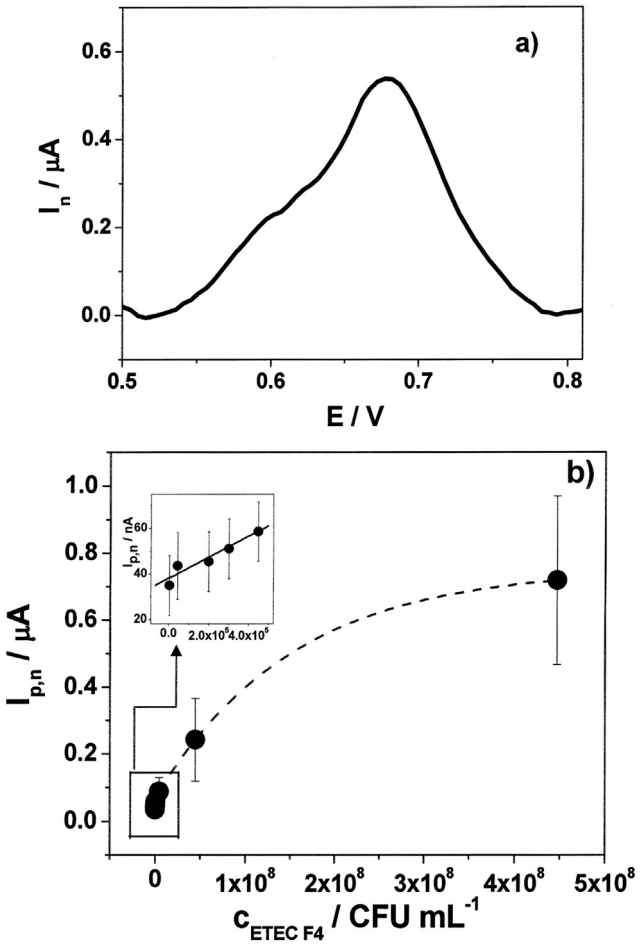

Fig. 6. a) Net currents of a square wave voltammogram of an ETEC *F4* suspension inactivated in autoclave. $c_{ETEC\ F4} = 4.72 \times 10^8$ CFU mL^{-1}. b) Calibration curve corresponding to inactivated ETEC *F4* suspensions. The dash line was plotted to show the trend.

Assuming the initial portion of the graph as a straight line (see insert of Fig. 6b), the linear regression ($n = 15$) is given by:

$$I_{p,n} = (3.3 \pm 0.9) \times 10^{-2}[\mu A] + (4.1 \pm 1.6) \times 10^{-8} c_{ETEC\ F4}\left[\mu A\ mL\ CFU^{-1}\right]; r = 0.9180$$

with a sensitivity of $(4.1 \pm 1.4) \times 10^{-8}$ μA mL CFU^{-1}. Experiments were done by triplicate. A limit of detection (LOD) $= 6.2 \times 10^5$ CFU mL^{-1} and a limit of quantitation (LOQ) $= 1.9 \times 10^6$ CFU mL^{-1} were estimated. They were calculated as [45]

$$LOD = \frac{3.3 S_{y/x}}{m}\sqrt{\left(1 + h_0 + \frac{1}{I}\right)}$$

and LOD = 3.03 LOD, where m is the slope of the calibration plot, I is the number of calibration samples and $S_{y/x}$is the residual standard deviation. h_0 is the leverage for the blank sample: $h_0 = \frac{\bar{c}^2 cal}{\sum_{i=1}^{I}(c_i - \bar{c}_{cal})^2}$ where $\bar{c}_{cal}$ is the mean calibration concentration and c_i is each of the calibration concentration values.

Clearly, a higher sensitivity (about ten folders) is obtained in inactivated ETEC *F*4 than in suspensions of live bacteria. The liberation of electroactive metabolites present in the inside of bacteria through thermal treatment for inactivation would be a possible explanation for this behavior.

3.4. Determinations in stool samples spiked with inactivated ETEC F4

The performance of the sensor was checked in stool samples. The electrochemical response obtained from diluted 1/5000 stool samples of pigs inactivated in autoclave is shown in Fig. 7 (line a). An increase in $I_{p,n}$ was clearly observed after the addition of ETEC *F*4 at a concentration of 2.00×10^7 CFU mL^{-1} (Fig. 7, line b).

Although the sensor is presented as nonselective, interferences in the current signal due to the presence of other bacteria (usually found in stool samples) and mainly from other molecules and/or macromolecules which are involved in cellular mechanisms, such as cytochrome *a*, *b* and *c*, ubiquinones and menaquinones, NADH, and succinate, are negligible in a 1/5000 dilution of swine feces. In addition, ETEC *F*4 determination was made using the method of standard additions. Thus, SWV measurements on this sample spiked with successive additions of ETEC *F*4 show that $I_{p,n}$ increases with the concentration of ETEC *F*4 in the 2×10^4–2×10^8 CFU mL^{-1} concentration range, as it was found with inactivated bacteria (Section 3.3).

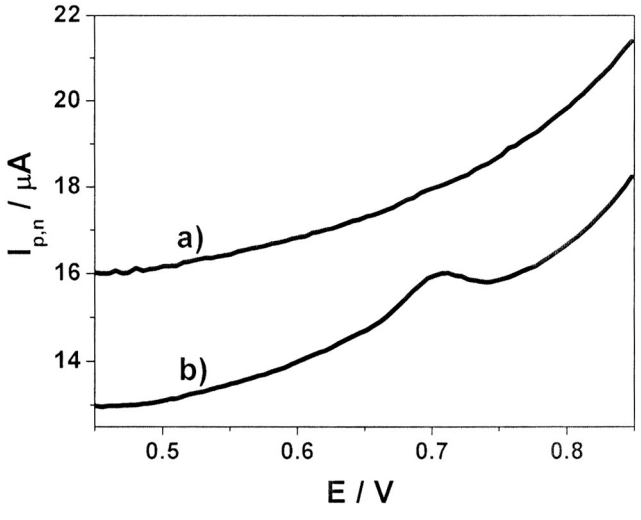

Fig. 7. a) Net currents of square wave voltammograms of a) 1/5000 dilution of swine feces sample in PBS; b) 1/5000 diluted sample spiked with 2×10^7 CFU mL^{-1} ETEC *F*4.

Experiments were done by triplicate. From the slope of the linear portion (interval 2×10^4–8×10^5 CFU mL^{-1}; r = 0.9980) of the three $I_{p,n}$ vs. $c_{ETEC\ F4}$ plots a relative standard deviation of about 20% was obtained. The sensitivity was $(8.08 \pm 0.09) \times 10^{-8}$ μA mL CFU^{-1} and a LOD $= 6 \times 10^4$ CFU mL^{-1} and a LOQ $= 2 \times 10^5$ CFU mL^{-1} were estimated.

Slightly higher sensitivity and slightly lower detection and quantification limits were obtained in stool samples spiked with inactivated ETEC *F*4 with respect to results of suspensions of pure inactivated ETEC *F*4 in PBS (Section 3.3). So, with this methodology it is possible the quantification of the ETEC *F*4 bacteria, inactivated by thermal lysis, in spiked diluted matrix of porcine feces.

As compared to other methods (see Table 1) this sensor shows a LOD higher than that of the fluorescent immunoassay, but close to that of PCR techniques, which is the most used for bacterial determination, with the advantage of a shorter analysis time and a lower cost, easy handling and it does not require isolation of ETEC *F*4 in a selective culture medium. On the other hand, a minimum of pre-treatment of the sample is necessary. Its use as screening tool is promissory.

4. Conclusions

An electrochemical sensor to detect and quantify the enterotoxigenic *E. coli* F4 (K88) bacteria in swine feces was developed. The sensor is based on a glassy carbon disk electrode modified with a dispersion of multi-walled carbon nanotubes in 1% Nafion™ aqueous solution. The ETEC *F*4 detection is performed through an irreversible oxidative signal by square wave voltammetry.

Samples of swine feces free of ETEC *F*4 and spiked with a pathogenic strain gave a detectable electrochemical signal by square wave voltammetry. This behavior was observed with both live bacteria and bacteria inactivated by thermal lyses. However, a significant increase in signal was observed for inactivated suspensions, possibly due to the presence of electroactive components of the bacteria liberated during the thermal pre-treatment.

The methodology developed is fast, precise, economical and suitable for direct electrochemical monitoring of the presence of ETEC *F*4 in swine samples without any pre-treatment. To the best of our knowledge, no reports exist in literature about electrochemical sensors for the determination of ETEC *F*4 in stool samples of pigs. Thus, the sensor

Table 1
Limit of detection of more relevant techniques for determination of ETEC *F*4.

Technique	Sample matrix	Detection limit	Ref.
Fluorescent immunosensor	PBS	1.1×10^3 CFU mL^{-1}	[46]
	Stool sample	2.1×10^3 CFU g^{-1}	
Multiplex real-time TaqMan PCR	Luria–Bertani broth	6.1×10^5 CFU mL^{-1}	[47]
Real time-PCR	Broth dilutions in PBS	F4ab 10^6, F4ac 10^5 and F4ad 10^4 CFU mL^{-1}[a]	[48]
	Stool sample without enrichment	F4ab 10^6, F4ac 10^6 and F4ad 10^4 CFU g^{-1}	
	Stool sample with enrichment	F4ab 10^3, F4ac 10^2 and F4ad 10^2 CFU g^{-1}	
Conventional PCR	Broth dilutions in PBS	F4ab 10^7, F4ac 10^6 and F4ad 10^6 CFU mL^{-1}	
	Stool sample without enrichment	Not detection	
	Stool sample with enrichment	F4ab 10^6, F4ac 10^4 and F4ad 10^4 CFU g^{-1}	
Fluorescent biosensor	PBS	1×10^2 CFU mL^{-1}	[49]
	Stool sample	2.5×10^2 CFU g^{-1}	
Electrochemical sensor	PBS	6.2×10^5 CFU mL^{-1}	This work
	Stool sample	6.0×10^4 CFU mL^{-1}	

[a] F4ab, F4ac and F4ad are variants of fimbriae *F*4.

proposed may be a tool capable of screening tests to determine the presence of ETEC *F*4 and thus determine points of infection early.

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References

[1] J.M. Fairbrother, E. Nadeau, C.L. Gyles, *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies, Anim. Health Res. Rev. 6 (2005) 17–39.
[2] B. Nagy, P.Z. Fekete, Enterotoxigenic *Escherichia coli* (ETEC) in farm animals, Vet. Res. 30 (1999) 259–284.
[3] F.A. Moredo, J.A. Cappuccio, L. Insarralde, C.J. Perfumo, M.A. Quiroga, G.A. Leotta, Caracterización genotipica de aislamientos de *Escherichia coli* obtenidos de cerdos con diarrea posdestete y enfermedad de los edemas, Rev. Argent. Microbiol. 44 (2012) 85–88.
[4] H.U. Bertschinger, Postweaning *Escherichia coli* diarrhea and edema disease, in: B.E. Straw, W.L. Mengeling, D.J. Taylor (Eds.), Diseases of Swine, Iowa State University Press, Ames 1999, pp. 441–454.
[5] P. Klemm, Fimbrial adhesins of *Escherichia coli*, Rev. Infect. Dis. 7 (1985) 321–340.
[6] G.M. Jensen, K. Frydendahl, O. Svendsen, C.B. Jørgensen, S. Cirera, M. Fredholm, J.-P. Nielsen, K. Møller, Experimental infection with *Escherichia coli* O149: F4ac in weaned piglets, Vet. Microbiol. 115 (2006) 243–249.
[7] C. Kaittanis, S. Santra, J.M. Perez, Emerging nanotechnology-based strategies for the identification of microbial pathogenesis, Adv. Drug Deliv. Rev. 62 (2010) 408–423.
[8] C.M. McEntegart, W.R. Penrose, S. Strathmann, J.R. Stetter, Detection and discrimination of coliform bacteria with gas sensor arrays, Sensors Actuators B Chem. 70 (2000) 170–176.
[9] M. Berrettoni, I. Carpani, N. Corradini, P. Conti, G. Fumarola, G. Legnani, S. Lanteri, R. Marassi, D. Tonelli, Coupling chemometrics and electrochemical-based sensor for detection of bacterial population, Anal. Chim. Acta 509 (2004) 95–101.
[10] M.S. Webster, J.S. Cooper, E. Chow, L.J. Hubble, A. Sosa-Pintos, L. Wieczorek, B. Raguse, Detection of bacterial metabolites for the discrimination of bacteria utilizing gold nanoparticle chemiresistor sensors, Sensors Actuators B Chem. 220 (2015) 895–902.
[11] L.M. Johnston, D. Elhanafi, M. Drake, L.-A. Jaykus, A simple method for the direct detection of *Salmonella* and *Escherichia coli* O157:H7 from raw alfalfa sprouts and spent irrigation water using PCR, J. Food Prot. 68 (2005) 2256–2263.
[12] S.D. Weagant, K.C. Jinneman, K.J. Yoshitomi, R. Zapata, W.M. Fedio, Optimization and evaluation of a modified enrichment procedure combined with immunomagnetic separation for detection of *E. coli* O157:H7 from artificially contaminated alfalfa sprouts, Int. J. Food Microbiol. 149 (2011) 209–217.
[13] C.H. Park, N.M. Vandel, D.L. Hixon, Rapid immunoassay for detection of *Escherichia coli* O157 directly from stool specimens, J. Clin. Microbiol. 34 (1996) 988–990.
[14] L. Wang, Y. Li, A. Mustapha, Rapid and simultaneous quantitation of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation, J. Food Prot. 70 (2007) 1366–1372.
[15] H.-Y. Jin, K.-H. Tao, Y.-X. Li, F.-Q. Li, S.-Q. Li, Microarray analysis of *Escherichia coli* O157:H7, World J. Gastroenterol. 11 (2005) 5811.
[16] S.M. Radke, E.C. Alocilja, A high density microelectrode array biosensor for detection of *E. coli* O157:H7, Biosens. Bioelectron. 20 (2005) 1662–1667.
[17] A. Wolter, R. Niessner, M. Seidel, Detection of *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Legionella pneumophila* in water using a flow-through chemiluminescence microarray readout system, Anal. Chem. 80 (2008) 5854–5863.
[18] L. Yang, Y. Li, G.F. Erf, Interdigitated array microelectrode-based electrochemical impedance immunosensor for detection of *Escherichia coli* O157:H7, Anal. Chem. 76 (2004) 1107–1113.
[19] X.-L. Su, Y. Li, A self-assembled monolayer-based piezoelectric immunosensor for rapid detection of *Escherichia coli* O157:H7, Biosens. Bioelectron. 19 (2004) 563–574.
[20] A. Subramanian, J. Irudayaraj, T. Ryan, A mixed self-assembled monolayer-based surface plasmon immunosensor for detection of *E. coli* O157:H7, Biosens. Bioelectron. 21 (2006) 998–1006.
[21] Y. Wang, Z. Ye, C. Si, Y. Ying, Subtractive inhibition assay for the detection of *E. coli* O157:H7 using surface plasmon resonance, Sensors 11 (2011) 2728–2739.
[22] T.B. Tims, D.V. Lim, Confirmation of viable *E. coli* O157:H7 by enrichment and PCR after rapid biosensor detection, J. Microbiol. Methods 55 (2003) 141–147.
[23] W. Wu, J. Zhang, M. Zheng, Y. Zhong, J. Yang, Y. Zhao, W. Wu, W. Ye, J. Wen, Q. Wang, An aptamer-based biosensor for colorimetric detection of *Escherichia coli* O157:H7, PLoS One 7 (2012) 1–9.
[24] R.Y. Hassan, H.N. Hassan, M.S. Abdel-Aziz, E. Khaled, Nanomaterials-based microbial sensor for direct electrochemical detection of *Streptomyces* spp, Sensors Actuators B Chem. 203 (2014) 848–853.
[25] S. Sirivisoot, Y. Parcharoen, T.J. Webster, Electrochemical detection of bacteria using graphene oxide electrodeposited on titanium implants, Adv. Sci. Tech. 96 (2014) 45–53.
[26] I. Carpani, P. Conti, S. Lanteri, P.P. Legnani, E. Leoni, D. Tonelli, Direct quantification of test bacteria in synthetic water-polluted samples by square wave voltammetry and chemometric methods, Biosens. Bioelectron. 23 (2008) 959–964.
[27] V. Mirceski, R. Gulaboski, M. Lovric, I. Bogeski, R. Kappl, M. Hoth, Square wave voltammetry: a review on the recent progress, Electroanal. 25 (2013) 2411–2422.
[28] X. Xiao, G. Zhu, L. Liao, B. Liu, Y. Yuan, Y. Wang, J. He, B. He, Y. Wu, A square wave voltammetric method for the detection of microorganism populations using a MWNT-modified glassy carbon electrode, Electrochim. Acta 74 (2012) 105–110.
[29] M. Valcarcel, B.M. Simonet, S. Cardenas, B. Suarez, Present and future applications of carbon nanotubes to analytical science, Anal. Bioanal. Chem. 382 (2005) 1783–1790.
[30] M. Trojanowicz, Analytical applications of carbon nanotubes: a review, Trends Anal. Chem. 25 (2006) 480–489.
[31] J.X. Wang, M.X. Li, Z.J. Shi, N.Q. Li, Z.N. Gu, Direct electrochemistry of cytochrome c at a glassy carbon electrode modified with single-wall carbon nanotubes, Anal. Chem. 74 (2002) 1993–1997.
[32] M.W. Musameh, J., A. Merkoci, Low-potential stable NADH detection at carbon-nanotube-modified glassy carbon electrodes, Electrochem. Commun. 4 (2002) 743–746.
[33] Laboratorio de Referencia de *E. coli* (LREC), Departamento de Microbiología y Parasitología, Facultad de Veterinaria, Universidad de Santiago de Compostela, Spain.
[34] W. Zhang, Progress and challenges in vaccine development against enterotoxigenic *Escherichia coli* (ETEC)-associated porcine postweaning diarrhoea (PWD), J. Vet. Med. Res. 1 (2014) 1006–1019.
[35] J. Osteryoung, J. Odea, Square-wave voltammetry, Electroanal. Chem. 14 (1986) 209–308.
[36] S. Subrahmanyam, K. Shanmugam, V.M. Madhav, M. Murugesan, T. Subramanian, D. Jeyakumar, Voltammetric studies of *Aspergillus niger*. Assessment of their growth using electroactive extracellular production of metabolite, Electroanal. 13 (2001).
[37] D.-M. Wu, G.-L. Fu, H.-Z. Fang, L. Hu, J.-L. Li, X. Yuan, Z.-Y. Zhang, Studies on the origin of the voltammetric response of the PC-3 cell suspension, Talanta 78 (2009) 602–607.
[38] Y. Chalenko, V. Shumyantseva, S. Ermolaeva, A. Archakov, Electrochemistry of *Escherichia coli* JM109: direct electron transfer and antibiotic resistance, Biosens. Bioelectron. 32 (2012) 219–223.
[39] D.R. Lovley, Electromicrobiology, Annu. Rev. Microbiol. 66 (2012) 391–409.
[40] R.Y. Hassan, U. Bilitewski, Direct electrochemical determination of *Candida albicans* activity, Biosens. Bioelectron. 49 (2013) 192–198.
[41] Y.-C. Zhang, Z.-H. Jiang, L. Ying, Application of electrochemically active bacteria as anodic biocatalyst in microbial fuel cells, Chin. J. Anal. Chem. 43 (2015) 155–163.
[42] S. Han, X. Li, G. Guo, Y. Sun, Z. Yuan, Voltammetric measurement of microorganism populations, Anal. Chim. Acta 405 (2000) 115–121.
[43] B. Kuznetsov, M. Khlupova, S. Shleev, A. Kaprel'yants, A. Yaropolov, An electrochemical method for measuring metabolic activity and counting cells, Appl. Biochem. Microbiol. 42 (2006) 525–533.
[44] J. Wang, Analytical Electrochemistry, third ed. J. Wiley & Sons, Hoboken, New Jersey, USA, 2006.
[45] A.C. Olivieri, Practical guidelines for reporting results in single- and multi-component analytical calibration: a tutorial, Anal. Chim. Acta 868 (2015) 10–22.
[46] Z. Peng, M. Ling, Y. Ning, L. Deng, Rapid fluorescent detection of *Escherichia coli* K88 based on DNA Aptamer Library as direct and specific reporter combined with immuno-magnetic separation, J. Fluoresc. 24 (2014) 1159–1168.
[47] D.M. West, K.A. Sprigings, C. Cassar, P.R. Wakeley, J. Sawyer, R.H. Davies, Rapid detection of *Escherichia coli* virulence factor genes using multiplex real-time TaqMan® PCR assays, Vet. Microbiol. 122 (2007) 323–331.
[48] J.W. Byun, B.Y. Jung, H.Y. Kim, J.M. Fairbrother, W.K. Lee, Real-time PCR for differentiation of F4 (K88) variants (F4ab, F4ac, F4ad) of enterotoxigenic *Escherichia coli* from diarrhoeic piglets, Vet. J. 193 (2012) 593–594.
[49] M. Ling, Z. Peng, L. Cheng, L. Deng, Rapid fluorescent detection of enterotoxigenic *Escherichia coli* (ETEC) K88 based on grapheme oxide-dependent nanoquencher and klenow fragment-triggered target cyclic amplification, Appl. Spectrosc. 69 (2015) 1175–1181.