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A novel strategy for screening-out raw milk contaminated with *Mycobacterium bovis* on dairy farms by double-tagging PCR and electrochemical genosensing

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Summary. A highly sensitive assay for rapidly screening-out *Mycobacterium bovis* in contaminated samples was developed based on electrochemical genosensing. The assay consists of specific amplification and double-tagging of the IS*6110* fragment, highly related to *M. bovis*, followed by electrochemical detection of the amplified product. PCR amplification was carried out using a labeled set of primers and resulted in a amplicon tagged at each terminus with both biotin and digoxigenin. Two different electrochemical platforms for the detection of the double-tagged amplicon were evaluated: (i) an avidin biocomposite (Av-GEB) and (ii) a magneto sensor (m-GEC) combined with streptavidin magnetic beads. In both cases, the double-tagged amplicon was immobilized through its biotinylated end and electrochemically detected, using an antiDig-HRP conjugate, through its digoxigenin end. The assay was determined to be highly sensitive, based on the detection of 620 and 10 fmol of PCR amplicon using the Av-GEB and m-GEC strategies, respectively. Moreover, the m-GEC assay showed promising features for the detection of *M. bovis* on dairy farms by screening for the presence of the bacterium's DNA in milk samples. The obtained results are discussed and compared with respect to those of inter-laboratory PCR assays and tuberculin skin testing. [**Int Microbiol** 2010; 13(2):91-97]

Keywords: *Mycobacterium bovis* · electrochemical DNA biosensor · avidin · magnetic beads · double-tagging PCR

Introduction

Tuberculosis (TB) in humans and other mammals is usually caused by infection with *Mycobacterium tuberculosis* or *Mycobacterium bovis*. Worldwide, *M. tuberculosis* is the single greatest cause of TB in humans, with the global prevalence of infection involving about one-third of the world's population and expected to increase steadily [2]. In most animals with bovine tuberculosis, *M. bovis* is the infective agent and the disease can be easily transmitted between farm animals. It is also a major zoonosis, mainly involving farm workers on dairy farms and the consumption of contaminated dairy products. Non-pasteurized milk is by far the most probable vehicle for the transmission of pathogenic mycobacteria, especially in developing countries where the prevalence of bovine TB is higher [3], and the isolation of *M. bovis* from milk samples of storage tanks, inadequately pasteurized

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milk, and milk samples from tuberculin non-reactive cattle has been reported [8]. As such, the detection of *M. bovis* in milk samples serves as an indirect diagnostic method—distinguishing infected from non-infected animals and controlling for airborne contamination with the bacilli—in order to prevent further spread of the disease.

To identify cattle infected with *M. bovis*, the intradermal tuberculin test is usually performed, which is based on the inoculation of *M. bovis* antigens called purified protein derivative (PPD). Although the tuberculin skin test is highly sensitive and specific, it requires 48–72 h to process, and veterinarians must be specially trained to perform the assay [4]. The culture of milk samples is another approach to the detection of *M. bovis*, but while it provides acceptable sensitivity and specificity it is labor-intensive, with up to 6 weeks required to detect positive specimens. Moreover, the low sensitivity of cultured milk has been reported, which can be attributed to the drastic pre-culture milk decontamination procedures and to the presence of mammary macrophages able to kill *M. bovis* bacilli [18]. More recent approaches to the rapid detection of *M. bovis* include chromatographic and molecular methods, such as PCR, which have advantages of speed, sensitivity, and specificity; however, they require adequately trained personnel and have high associated costs (reagents and equipment) [17]. Biosensors, by contrast, offer an exciting alternative, allowing the rapid and multiple analyses essential for the detection of bacteria in food [5]. Consequently, they are of particular interest for developing countries, where contaminated milk remains an important issue. Biosensors are devices based on the combination of biological receptors (mainly antibodies, enzymes, nucleic acids, whole cells) and physical or physicochemical transducers. In most cases, they allow "real-time" observations of specific biological events (e.g., antibody-antigen interaction) as well as the detection of a broad spectrum of analytes in complex sample matrices. In the literature, a few assays for *M. bovis* detection have been described that are based on optical and piezoelectric biosensors or on a gas sensor array [1,7,9,15]. These devices, although less robust, are more user-friendly, portable, and cost-effective than electrochemically based transduction devices. Furthermore, electrochemical biosensors can operate in turbid media and offer enhanced sensitivity.

To our knowledge, the present report is the first description of an electrochemical strategy for the rapid screening-out of raw milk contaminated with *M. bovis*, using a procedure based on electrochemical genosensing. The insertion fragment IS*6110*, highly related to *M. bovis* [16–18], was amplified by double-tagging PCR using a set of primers labeled with biotin and digoxigenin, respectively. During PCR amplification of the *M. bovis* insertion fragment, the amplicon ends were double-tagged with (i) the biotinylated capture primer, to achieve immobilization on the genosensing transducer, and (ii) the digoxigenin signaling primer, to allow enzymatic detection through the antiDigG-horseradish peroxidase (HRP) reporter. The genosensing transducer was immobilized by using a highly specific biocomposite bulk-modified with the protein avidin (Av-GEB) [14] or, alternatively, streptavidin-modified magnetic beads to achieve improved retention of the beads on a highly sensitive magneto sensor (m-GEC) [6,13]. In this report, the features of both electrochemical assays are discussed and compared with respect to inter-laboratory PCR assays and the tuberculin screen-out skin test, the current gold standard for identifying cattle exposed to *M. bovis*.

Materials and methods

Instrumentation. Amperometric measurements were performed with a LC-4C amperometric controller (BAS Bioanalytical Systems, USA). A three-electrode setup was used, comprising a platinum auxiliary electrode (Crison 52-67 1, Spain), a double junction Ag/AgCl reference electrode (Orion 900200) with 0.1 M KCl as the external reference solution, and a working electrode (m-GEC or Av-GEB electrode) [6b]. The amperometric signals were registered using a DUO-18 data recording system (WPI, UK). Temperature-controlled incubations were done in an Eppendorf compact thermomixer. PCR was carried out in an Eppendorf Mastercycler Personal thermocycler. Magnetic beads were magnetically separated using a Dynal MPC-S magnetic separator (prod. no. 120.20, Dynal Biotech ASA, Oslo, Norway).

Chemicals and biochemicals. The graphite-epoxy composite and biocomposite were prepared using 50-μm particle size graphite powder (BDH, UK) and Epo-Tek H77 epoxy resin and hardener (both from Epoxy Technology, Billerica, MA, USA). The Av-GEB biocomposite was prepared with avidin (prod. no. A9275, Sigma, Steinheim, Germany). The streptavidin-modified magnetic beads with Dynabeads M-280 and streptavidin (prod. no. 112-05D, Dynal Biotech ASA). Fab fragments of anti-digoxigenin-POD (prod. no. 1207733) were used as enzyme reporter and were purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Two primers 20-nucleotides long were obtained from TIB-MOLBIOL (Berlin, Germany) and designed for PCR amplification of the insertion sequence IS*6110*, related to *M. bovis* [18]. The primer sequences were: biotinylated IS*6110* up: 5′ bio-GCG TAG GCG TCG GTG ACA AA-3′ and digoxigenated IS*6110* down: 5′ dig-CGT GAG GGC ATC GAG GTG GC-3′. The Expand High Fidelity PCR System Kit (Roche Molecular Biochemicals) was used for the PCR.

All other reagents were of the highest available grade. Aqueous solutions were prepared with Milli-Q water. The compositions of these solutions were as previously described [6b].

DNA amplification and double tagging for the electrochemical detection of *M. bovis*. Raw milk samples were collected from local dairy farm tanks and transported refrigerated to the laboratory. The samples were deactivated at 70°C for 70 min and stored at –20°C until they were used. A 125-ml volume of the sample was centrifuged at 3000 rpm for 15 min, and the cell layer obtained was washed in PBS and resuspended in 1 ml of PBS. The cellular suspension was diluted 1:2 in NTE buffer with 10% SDS, incubated at 37°C for 1 h, and then overnight at 37°C with 1% proteinase K. DNA was purified by two extractions with phenol:chloro-

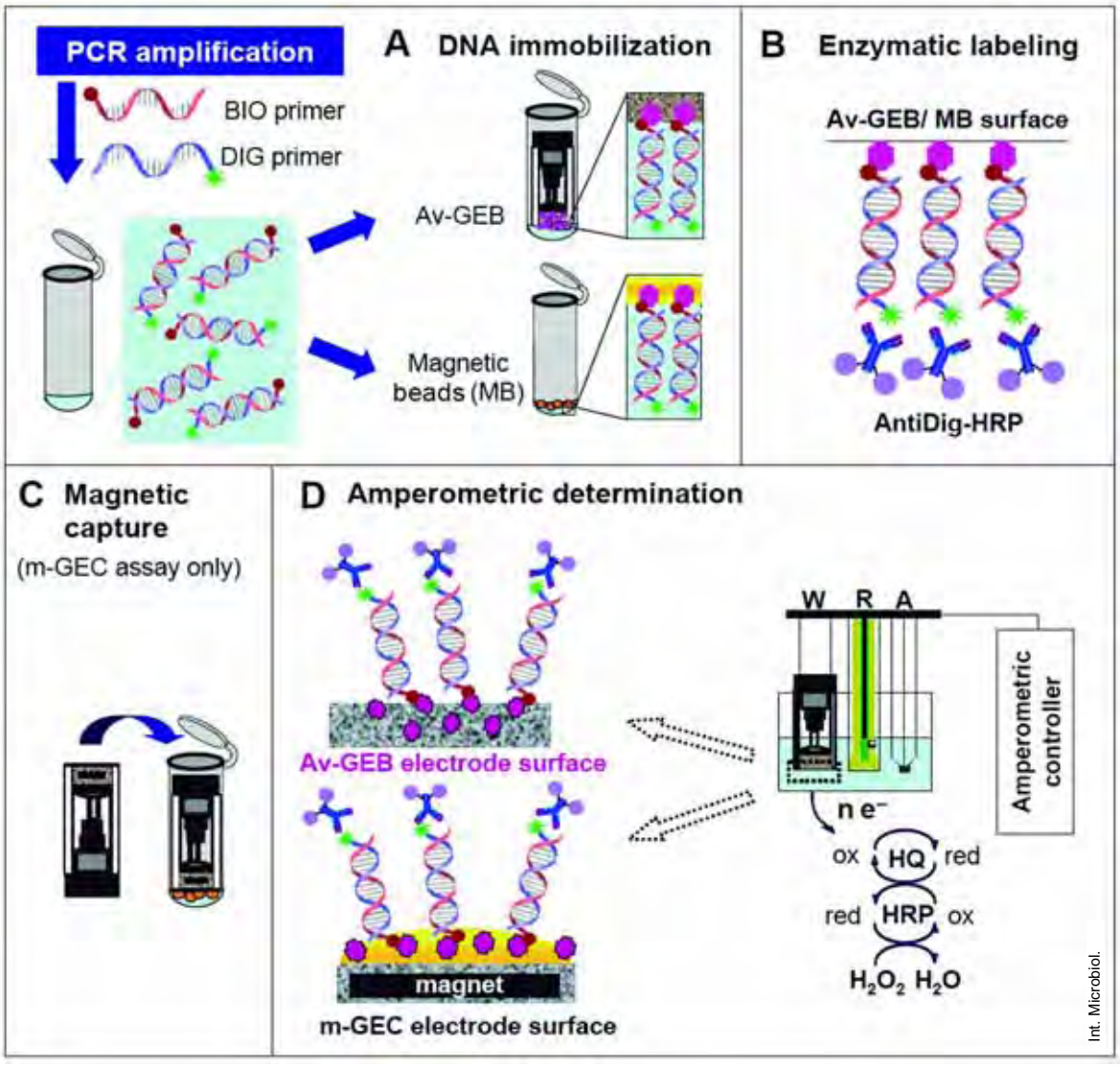

Fig. 1. Schematic representation of the electrochemical strategy for the detection of *Mycobacterium bovis*. For details, see text.

form:isoamyl alcohol (25:24:1) and one with chloroform:isoamyl alcohol (24:1), collected by precipitation with 5 M NaCl and isopropanol, and kept overnight at –20°C. The precipitated DNA was then washed with 1 ml of ethanol 70° and resuspended in 40 µl of RNase-free water.

As shown in Fig. 1A, a primer pair tagged with biotin and digoxigenin, respectively, was used for amplification and double-tagging of the PCR amplicon. PCR was performed in a 100-µl reaction mixture containing 8 µl of purified DNA isolated from *M. bovis*. Each reaction contained 100 µM of each deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP), 0.1 µM of the double-tagged set of primers (biotinylated IS*6110* up and digoxigenated IS*6110* down), and 5.6 U of polymerase. The reaction was carried out in Expand High Fidelity 1× buffer (Roche), containing 1.5 mM $MgCl_2$ and 5% v/v dimethyl sulfoxide (DMSO). The amplification mixtures were exposed to an initial step at 95°C for 2 min followed by 30 cycles at 95°C for 30 s, 64°C for 30 s, and 72 ºC for 30 s, and a last step of 7 min at 72°C. The resulting samples were stored at 4°C.

All of the amplifications included a negative control, which contained all reagents, except *M. bovis* template, in the PCR mixture. The amplification products were analyzed by electrophoresis on 2% agarose gel in TAE buffer (0.04 M Tris, 0.1% v/v acetic acid, 1 mM EDTA, pH 8.0), containing 0.5 µg ethidum bromide/ml. A *Hin*fI-digested ϕX174 genome consisting of DNA fragments ranging in size from 24 to 726 bp was used as a DNA size marker. The DNA bands were visualized by UV trans-illumination. As the primers were labeled with biotin and digoxigenin, the amplicon was expected to be double-tagged with both biotin and digoxigenin at each terminus.

Construction of the magneto graphite-epoxy composite (m-GEC) and avidin graphite-epoxy composite (Av-GEB) electrodes. The m-GEC and Av-GEB electrodes were designed in our laboratories. The detailed preparation was extensively described by Pividori et al. [12] and was based on a rigid graphite-epoxy composite [10,11]. For the Av-GEB electrode, avidin was hand mixed with the graphite power and epoxy resin paste, resulting in a 1% (w/w) bulk-modified biocomposite. The magneto electrodes based on GEC as well as those based on the biocomposite material were cured at 40°C for one week. Prior to each use, the electrode surface was renewed by a simple polishing procedure, i.e., wetted with doubly distilled water, and then thoroughly smoothed with abrasive paper and finally with alumina paper [6].

Electrochemical genosensing of the double-tagged *M. bovis* amplicon using Av-GEB electrodes. Electrochemical detection based on the Av-GEB electrodes consisted briefly of the following steps (Fig. 1): (i) immobilization of the double-tagged amplicon on the Av-GEB electrode (Fig. 1A), with the biotin-tagged terminus of the dsDNA amplicon attached to the surface of the electrode; (ii) enzymatic labeling using antiDig-HRP, which attaches to the 3′ digoxigenin end of the amplicon (Fig. 1B); (iii) amperometric determination (Fig. 1D).

After the amplification, the PCR amplicon was diluted in Milli-Q water and 10 µl were incubated in 5× SSC for 15 min at 42°C. The double-tagged amplicon was then immobilized by dipping the Av-GEB electrode into an Eppendorf tube containing the diluted amplicon. Immobilization was carried out in 5× SSC solution at a final volume of 140 µl for 30 min at 42°C. The prepared electrode was then washed twice with 140 µl of 5× SSC for 10 min at 42°C. In the next step, the immobilized amplicon was enzymatically labeled for 30 min at 42°C using antiDig-HRP (60 µg) in a reaction containing Tris blocking buffer and a final volume of 140 µl. The immobilized, enzymatically labeled amplicon was then washed twice for 10 min at 42°C in 140 µl of Tris buffer. Electrochemical determination was carried out using the modified Av-GEB electrode as working electrode and by dipping the three-electrode setup (described in Materials and methods) in 20 ml of phosphate buffer. The response was determined by polarizing the electrodes at –0.150 V (vs. Ag/AgCl). Amperometric detection was based on the activity of the HRP enzyme as electrochemical reporter, using 1.81 mM hydroquinone as the mediator and 4.90 mM hydrogen peroxide as the substrate for the enzyme HRP.

Electrochemical genosensing of the double-tagged *M. bovis* amplicon using m-GEC electrodes. Electrochemical detection based on m-GEC electrodes consisted briefly of the following steps (Fig. 1): (i) immobilization of the double-tagged amplicon on streptavidin magnetic beads, with the 5′ biotin end immobilized on the beads (Fig. 1A); (ii) enzymatic labeling using antiDig-HRP, which attaches to the 3′ digoxigenin end of the amplicon (Fig. 1B); (iii) magnetic capture of the modified magnetic particles by the m-GEC electrode (Fig. 1C); (iv) amperometric determination (Fig. 1D).

As was done in the Av-GEB procedure, following amplification, the PCR amplicon was diluted in Milli-Q water and 10 µl were incubated in 5× SSC for 15 min at 42°C. The double-tagged amplicon was then immobilized by adding 6.5×10^6 streptavidin magnetic beads to an Eppendorf tube containing the diluted amplicon. Immobilization was carried out in 5× SSC solution at a final volume of 140 µl for 30 min at 42°C. The subsequent washing steps and electrochemical detection were the same as described for the Av-GEB platform.

Results

DNA amplification and double tagging for electrochemical detection of *M. bovis*. As shown in Fig. 2, under the PCR conditions used here, the double-tagged set of primers exclusively amplified IS*6110*. Figure 2 also shows no bands in the negative PCR control sample, which included all reagents except the DNA template.

Electrochemical genosensing of the double-tagged *M. bovis* amplicon using Av-GEB and m-GEC electrodes. In Fig. 3A, the responses obtained with the different dilutions of double-tagged IS*6110* PCR amplicon using the Av-GEB (1/15, 1/8, 1/4, and 1/2) and m-GEC (1/960, 1/480, 1/240, 1/120, 1/60, 1/30, 1/15, 1/8, and 1/4) electrodes are plotted against the PCR amplicon concentration determined spectrophotometrically at 260 nm. The electrochemical signals were obtained under conditions at which the enzyme was saturated with the substrate. For each measurement, a steady-state current was obtained after the addition of hydroquinone and hydrogen peroxide (normally after 1 min of addition of the latter), as shown in Fig. 3B. This steady-state current was also used for the electrochemical signal plotted in Fig. 4. As shown in Fig. 3A, the analytical response of both electrodes increased quantitatively with the amount of double-tagged amplicon, but the sensitivity of the assay based on the m-GEC electrodes (black line) was higher than that obtained with the Av-GEB electrode (dotted line). The inset in Fig. 3A shows in detail the responses obtained with the two electrodes at the lowest concentration range. The lowest amount of analyte producing a meaningful analytical signal was 620 fmol for the Av-GEB electrode and 10 fmol for the m-GEC electrode.

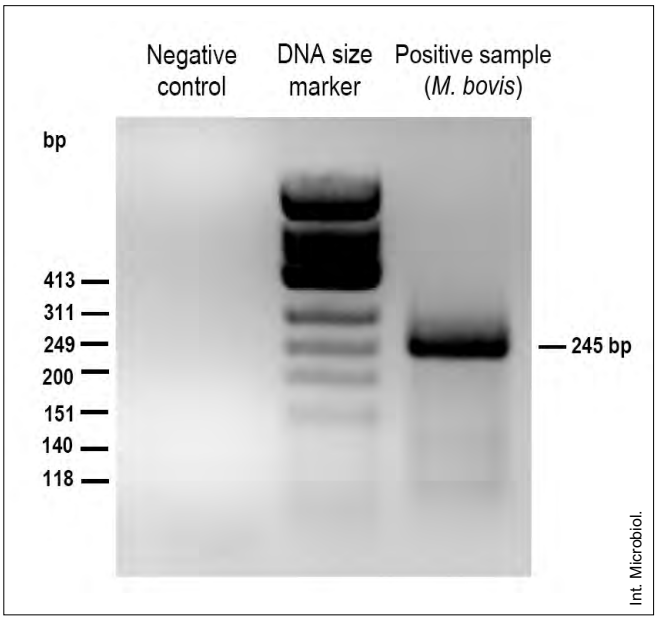

Fig. 2. Gel electrophoresis of the DNA amplification product using the double-tagging set of primers.

Figure 4 shows the electrochemical response provided by five different milk samples from dairy farms using double-tagging PCR combined with either the Av-GEB or the m-GEC strategy, as an indicator of infected cattle. In order to screen-out negative samples, a cut-off value was established by using both electrochemical genosensing strategies to analyze a negative milk sample (as confirmed by two inter-laboratory PCR assays and by the tuberculin skin test). Accordingly, four replicates of the negative control were

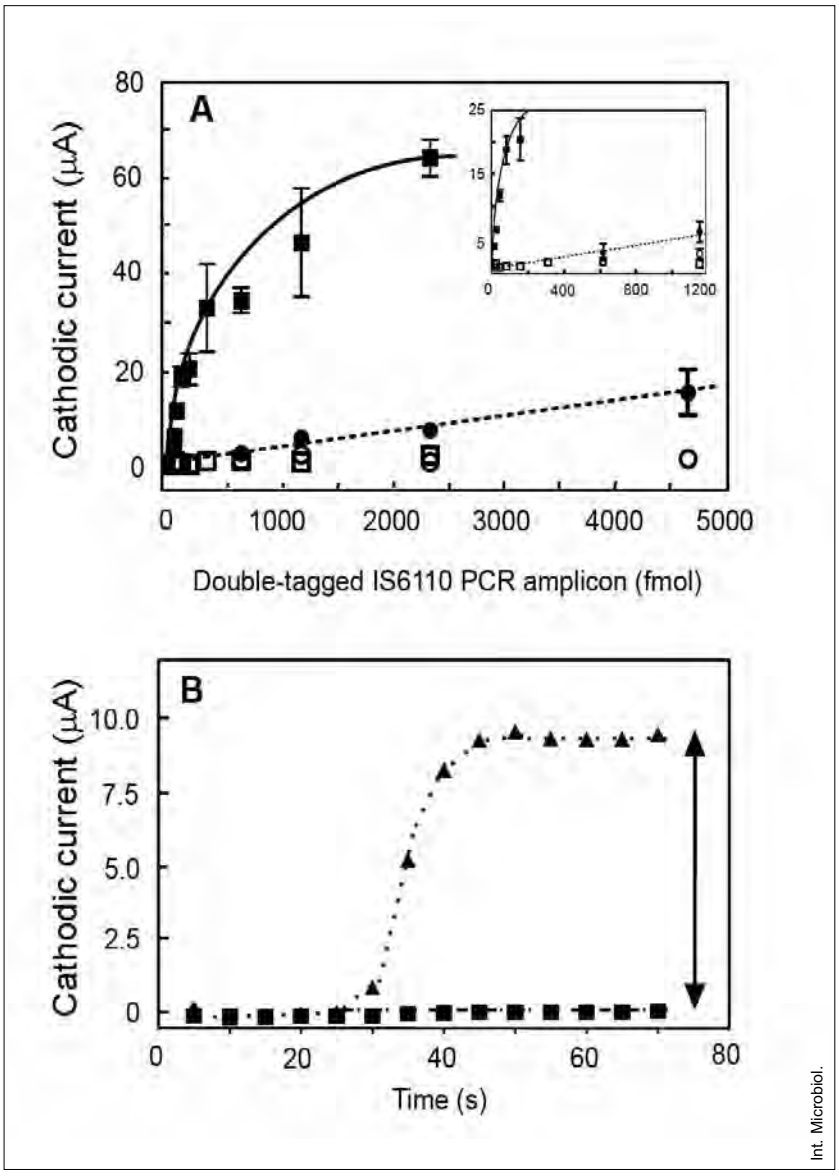

Fig. 3. (**A**) Electrochemical detection of the *Mycobacterium bovis* PCR product based on the Av-GEB and m-GEC strategies. Closed circles, positive sample Av-GEB; open circles, negative control Av-GEB; closed squares, positive *M. bovis* sample m-GEC; open squares, negative control m-GEC. (**B**) The typical amperometric curve, showing the enzyme saturation signal. Closed triangles, positive *M. bovis* sample; closed squares, negative control. The dotted line shows the cut-off value. In all cases (A and B), n = 3.

processed, obtaining a mean value of 1.4 μA with a standard deviation of 0.8 μA for the Av-GEB assay and a mean value of 3.3 μA with a standard deviation of 0.3 μA for the m-GEC assay. The cut-off value was then extracted using a one-tailed *t* test at a 95% confidence level, giving a value of 3.9 μA and 4.2 μA for the Av-GEB and m-GEC strategies, respectively (shown in Fig. 4 as a dotted line). The assay based on the m-GEC electrode showed the presence of *M. bovis* in samples 3, 4, and 5, whereas only sample 5 was positive in the Av-GEB assay (3 and 4 gave negative results). The inset of Fig. 4 shows in detail the responses obtained for samples 3 and 4.

These results were compared with those obtained in analyses of the milk samples by inter-laboratory PCR assays and administering the tuberculin skin test to the animals (Table 1).

Discussion

DNA amplification and double tagging for the electrochemical detection of *M. bovis*. To our knowledge, this is the first report in which double-tagging PCR was carried out for the detection of *M. bovis*. Both the biotin and the digoxigenin moieties could be successfully incorporated into the PCR product using a set of 5′ labeled primers, as confirmed in Fig. 2. After annealing of both 5′ labeled primers with the template, a new DNA strand was enzymatically assembled by the Taq polymerase, by the addition of nucleotides to the 3′ end of both primers. The primers, and thus their tags, were included in the amplicon.

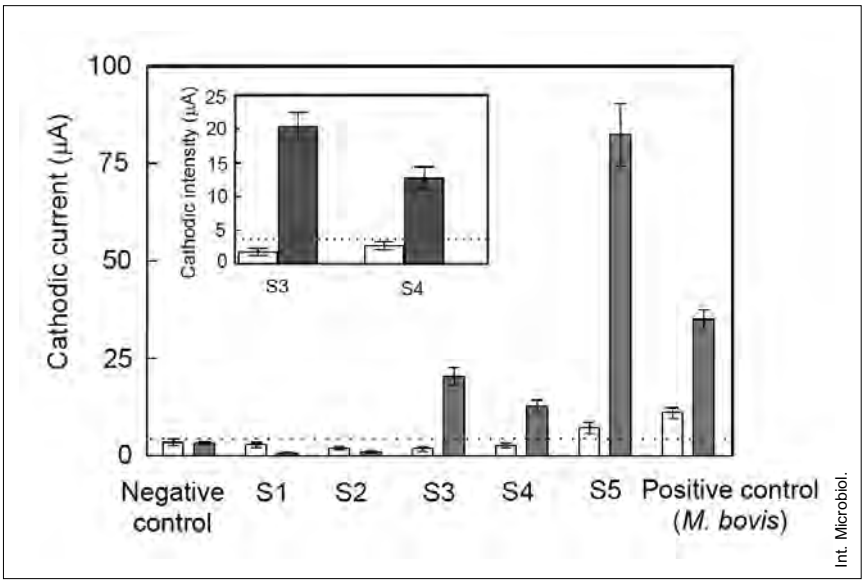

Fig. 4. Detection of *Mycobacterium bovis* in milk samples using the electrochemical detection based on the Av-GEB (white bars) and m-GEC (gray bars) strategies. The dotted line shows the cut-off value, n = 3. (S1, sample 1; S2, sample 2; S3, sample 3; S4, sample 4; S5, sample 5.)

Electrochemical genosensing of the double-tagged amplicon of *M. bovis* using Av-GEB and m-GEC electrodes. In the electrochemical genosensing procedure based on the Av-GEB electrodes, the double-tagged amplicon was immobilized on the surface of the avidin biocomposite transducer, while in the electrochemical genosensing procedure based on the m-GEC electrodes, the double-tagged amplicon was immobilized on streptavidin magnetic beads and then captured on the surface of the magneto electrode (m-GEC). In both cases, the electrochemical response of the double-tagged product was due to the enzymatic reporter antiDig-HRP. As shown in Fig. 3, the electrochemical genosensing strategy based on m-GEC had a higher sensitivity, obtained by using streptavidin magnetic beads, which immobilized the biotinylated amplified material on the m-GEC surface and permitted rapid magnetic separation of the unbound components. However, non-specific adsorption for both electrodes, as determined with the negative PCR control, was low and almost the same throughout the evaluated concentration range. The results showed that both strategies, using Av-GEB and m-GEC electrodes, were suitable for the detection of amplified PCR amplicon, although a better limit of detection (LOD) was achieved with (strept)avidin magnetic beads coupled with m-GEC electrodes.

The high specificity of the tuberculin skin test (96%, i.e., the proportion of negatives that are correctly identified) ensures the correct identification of a negative sample. Nevertheless, the test is unable to ensure the total absence of *M. bovis* in milk samples, as its sensitivity is only 86% [4]. Since screening assays are used on large sample populations, often with the aim of determining which samples require further investigation, false-positives are not as problematic as false-negatives, since the former will be further examined.

Due to the high specificity of the tuberculin skin test (96%), samples 3 and 4 (Fig. 4), with positive tuberculin skin test results (Table 1), in all likelihood came from infected animals. However, inter-laboratory PCR assays as well as

Table 1. Results of inter-laboratory PCR assays and tuberculin skin tests of milk samples screened for *Mycobacterium bovis*

	Inter-laboratory PCR assays			Electrochemical assays	
Sample	Lab1	Lab2	Tuberculin skin test	m-GEC	Av-GEB
1	Positive	Positive	Negative PPD	Negative	Negative
2	Positive	Positive	Negative PPD	Negative	Negative
3	Negative	Positive	Positive PPD	Positive	Negative
4	Negative	Negative	Positive PPD	Positive	Negative
5	Positive	Negative	Negative PPD	Positive	Positive

electrochemical genosensing of the double-tagged amplicon based on the Av-GEB platform gave false-negative results for these samples. As a false-negative can be the source of misdiagnoses, with severe consequences, the poor analytical performance in screening-out negative samples is noteworthy. By contrast, positive results, consistent with the tuberculin skin tests (Table 1), were obtained for samples 3 and 4 by electrochemical genosensing of the double-tagged amplicon using the m-GEC platform (Fig. 4).

The discrepancy in the electrochemical genosensing results was likely due to the fact that the m-GEC approach has a better LOD than the Av-GEB approach (10 vs. 620 fmol of double-tagged amplicon, respectively), allowing the identification of samples 3 and 4 as positive with higher sensitivity.

The negative results obtained for sample 5 with the tuberculin skin test but not by electrochemical genosensing with either the m-GEC or the Av-GEB electrodes (as displayed in Fig. 4 and Table 1) could be ascribed to the lower sensitivity of the traditional test. Accordingly, sample 5 should be further investigated. However, as the primary use of electrochemical genosensing of the double-tagged amplicon based on m-GEC is to screen-out negative samples, the most important parameter is the LOD, and thus to consider any negative results as definitive. By contrast, positive test results always should be considered presumptive and must be confirmed by an approved culture method.

Electrochemical genosensing with m-GEC electrodes shows interesting analytical features suggesting this approach as a promising strategy to screen-out negative dairy samples and thereby to isolate negative cattle from presumptive infected animals. The combination of genome amplification by double-tagging PCR, capture of the double-tagged amplicon, and electrochemical genosensing detection using the sensitive m-GEC electrode provides a rapid, cheap, and sensitive assay for the screening-out of samples contaminated with *M. bovis*.

Future work will be focused on the analytical validation of this promising electrochemical genosensor by processing a higher number of dairy samples. In addition, the modification of this methodology to include disposable, low-cost screen-printed electrodes is of great interest.

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