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OLED-based DNA biochip for *Campylobacter* spp. detection in poultry meat samples

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ABSTRACT

Integrated biochips are the ideal solution for producing portable diagnostic systems that uncouple diagnosis from centralized laboratories. These portable devices exploit a multi-disciplinary approach, are cost effective and have several advantages including broader accessibility, high sensitivity, quick test results and ease of use. The application of such a device in food safety is considered in this paper. Fluorescence detection of a specific biological probe excited by an optical source is one of the most commonly used methods for quantitative analysis on biochips. In this study, we designed and characterized a miniaturized, highly-sensitive DNA biochip based on a deep-blue organic light-emitting diode. The molecular design of the diode was optimized to excite a fluorophore-conjugated DNA probe and tested using real meat samples to obtain a high sensitivity and specificity against one of the most common poultry meat contaminants: *Campylobacter* spp. Real samples were analyzed also by classical plate methods and molecular methods to validate the results obtained by the new DNA-biochip. The high sensitivity obtained by the OLED based biochip (0.37 ng/μl) and the short time required for the results (about 24 h) indicate the usefulness of the system.

1. Introduction

Campylobacteriosis is considered the most frequent foodborne illness in the European Union (EU), and the most common food contaminated by *Campylobacter* is chicken meat. One of the most important goal in food security is the development of accurate and early diagnosis for foodborne diseases. The European Food Safety Authority (EFSA) estimated that approximately nine million cases of campylobacteriosis occur each year in Europe, with a cost to public health systems of approximately EUR 2.4 billions.

Both classical culture media based and molecular biology techniques used for a long time have limitations: the long time required by the classical methods to grow microorganisms, that can lead to the distribution of contaminated food, and the sensitivity to contaminants of DNA polymerase used in PCR.

Moreover, some bacteria can be stressed by food industry heat treatments and are not able to grow on selective agar plates, (viable but-not culturable, VBNC), thus making plate count methods unsuitable for testing. Molecular biology has greatly improved the techniques by reducing the time required to obtain results. Although real-time-PCR (Levi et al., 2003) allows results to be obtained in a few hours, the inhibition caused by contaminants in the DNA polymerase used in PCR, can produce false-negative results. Moreover, PCR tests are normally run in a laboratory context, while the opportunity to perform point-of-care food controls can improve the safety of food distribution. Recent advances in biosensor technology promise sensitive and specific point-of-care tests with rapid results.

"Different detection technologies have been used in the development of biosensors that can be used for the rapid screening of foods to detect foodborne pathogens prior to distribution, like for example optical sensors (Passaro et al., 2012), acoustic sensors (Jia et al., 2012), microwire sensors (Lu and Jun, 2012) and electrochemical biosensors (Marks et al., 2007)".

Antibodies, cells and DNA have been used as probes in the fabrication of biosensors (Lei et al., 2006). In particular, DNA is a biological element that is useful for the creation of genosensors (DNA-biosensors) (Cecchini et al., 2012), which allow the rapid monitoring of hybridization with the target DNAs. These biosensors, which are based on the oligonucleotide sequences chosen as probes, are specific and sensitive. To reveal the presence of a hybrid generated by the annealing of the DNA probe to the DNA target in various samples, it is

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possible to use a detected a fluorescent signal. In particular, DNA probes can be labelled with fluorophores, and their weak optical signal can be detected using a charge-coupled device (CCD) camera after a suitable optical excitation.

Various authors (Yao et al., 2005; Hofmann et al., 2005; Pais et al., 2008; Ramuz et al., 2009; Lamprecht, 2010) demonstrated the utilization of an organic light emitting device (OLED) source as a fluorescence excitation source to produce a sensitive biochip.

In particular, the use of an optimized OLED source for the detection of protein arrays has been demonstrated by Marcello et al. (2013). In this work we apply, this OLED light source to a DNA-biochip for the detection of *Campylobacter,* one of the most important pathogens responsible for human gastroenteritis. Campylobacteriosis still causes large economic losses worldwide. Classical methods for *Campylobacter* identification in food samples rely on broth enrichment and colony growth on selective agar plate which takes, at least five days (ISO 10272-1B: 2006) (Voedsel en Warent Autoriteit, 2010). In this work, we tested the sensitivity of a new bio-sensor using both pure culture reference strains and real poultry meat samples to determine the sensitivity of the biochip. The tests results are compared with the standardised laboratory methods including PCR, broth enrichment and colony growth, to demonstrate the relevance of this system for a rapid, simple and reliable point-of-care test for poultry meat.

2. Materials and methods

2.1. OLED fabrication

For this experiment high quality polished borosilicate glass substrates of 1 mm thick coated with 150 nm of indium tin oxide (ITO) of about 20 Ω/square surface resistance have been used. The ITO has been partially removed by a lithographic process using UV curable resins and a mask aligner in a class-10 clean room. Before coating the samples with poly(3,4-ethylenedioxythiophene)-poly (styrenesulfonate) (PEDOT-PSS), an ultrasonic cleaning with organic solvents (acetone, iso-propanol and ethyl alcohol) and demineralized water has been performed on each substrate. All samples have then been dried with nitrogen. After cleaning, the PEDOT-PSS was deposited on the ITO glass substrate at a speed of 2000 RPM for 20 s in the clean room. After the PEDOT-PSS coating, the samples were annealed at 100 °C in air atmosphere for 5 min. After this treatment the samples were transferred into a BOC EDWARDS 500 evaporator, integrated in a pure nitrogen filled JACOMEX glove box, for both the organic layers and the metal cathode deposition. For the organic layers deposition Knudsen cells, each integrated with a thermocouple, for a PID (proportional integral derivative control) setting and a constant monitoring of the cell temperature, have been used. The deposition rate of the organic layers was set at 0.1 nm/s and the film thickness was monitored, during the evaporation, by a calibrated quartz microbalance. A thin LiF layer (1 nm) and a pure aluminium (99.99%) layer was then deposited by electron beam technique in the same BOC EDWARDS 500 evaporator. Two different evaporation rates of 0.01 nm/s and 0.2 nm/s were used for the thin films evaporation, and the film thickness has been monitored by a calibrated quartz microbalance. During both the organic and metal evaporations the pressure in the vacuum chamber was maintained at 1×10^{-6} mbar. After the evaporation the organic light emitting diode (OLED) samples have been encapsulated with a glass lid and a UV curable resin in the glove box. The optical and electrical characterization of the OLED samples was performed in air. The spectral emission and the radiance of the OLED device, measured at normal incidence, was recorded with a GL Spectis 5.0 spectroradiometer (GL Optics GmbH), while the *J–V* curves of the OLED device were recorded with a source metre specifically developed ain the LAPLACE laboratory (Toulouse, France).

Table 1
Reference microorganisms used to test sensitivity and specificity of the DNA probes.

No.	Microorganism	Source
1	*Weissella cibaria*	DSM 14295[a]
2	*Vibrio spp.*	DSM 14379[a]
3	*Escherichia coli*	DISTAM[b]
4	*Pseudomonas aeruginosa*	DISTAM[b]
5	*P. migulae*	DISTAM[b]
6	*P. fluorescens*	DISTAM[b]
7	*P. brennerii*	DISTAM[b]
8	*Bacillus coagulans*	DSM 2308[a]
9	*B. subtilis*	DSM 1029[a]
10	*B. cereus*	DSM 2301[a]
11	*Proteus vulgaris*	DISTAM[b]
12	*Yersinia enterocolitica*	DISTAM[b]
13	*Morganella morganii*	DISTAM[b]
14	*Salmonella Enteritidis*	DSM 4883[a]
15	*Listeria monocytogenes*	ATCC 7644[c]
16	*Citrobacter freundii*	DSM 15979[a]
17	*Enterobacter cloacae*	DSM 30054[a]
18	*Aeromonas sobria*	DSM 19176[a]
19	*Lactobacillus plantarum*	DSM 20174[a]
20	*Pediococcus pentosaceus*	DSM 20336[a]
21	*Leuconostoc lactis*	CECT 4173[d]
22	*Saccharomyces cerevisiae*	ATCC 36024[c]
23	*Campylobacter jejuni subsp. jejuni*	DSM 4688[a]
24	*C. jejuni*	ATCC BAA-1153[c]
25	*C. jejuni*	ATCC 49943[c]
26	*C. coli*	DSM 24155[a]
27	*C. coli*	DSM 24128[a]
28	*C. coli*	ATCC 43478[c]
29	*C. lari subsp. lari*	DSM 11375[a]
30	*C. upsaliensis*	DSM 5365[a]
31	*Helicobacter pylorii* p1	Hospital of Udine[e]
32	*Helicobacter pylorii* p2	Hospital of Udine[e]

[a] DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany).
[b] DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (Milan, Italy).
[c] ATCC: American Type Culture Collection (Manassas, VA, USA).
[d] CECT: Colección Española de Cultivos Tipo (University of Valencia, Spain).
[e] Isolated from hospitalized patient (Hospital of Udine, Italy).

2.2. Strains selection and DNA preparation

As a first step for the bio-chip construction, 32 microorganisms (31 bacteria and 1 yeast from international collections) listed in Table 1 were used for testing the specificity and sensitivity.

To evaluate the specificity of the designed probes the standardization of the DNA extracted from the different bacteria listed in Table 1 is necessary.

The DNA of the reference strains was extracted and purified from one millilitre of overnight broth culture using the Wizard® Genomic DNA Purification Kit (Promega, Milan, Italy) (Cecchini et al., 2012). The purity and concentration of the DNA samples were evaluated by spotting 1 µl of the extracted DNA onto the spectrophotometer nanodrop 2000c (Thermo Fisher Scientific, Wilmington, DE, U.S.A.) which allow the measure of the DNA avoiding dilution steps. After the reading of the concentrations the samples were standardized at the same concentration using ddwater.

2.3. DNA probe construction and test

A new 55-base DNA detection probe (CampyDet) (5′ CACTTTTCGGAGCGTAAACTCCTTTTCTTAGGGAAGAATTCTGACGGTA CCTAAG-3′) specific for the 16 S rRNA gene of *Campylobacter* spp.

was designed, to be used with the 45-base capture probe (5′-GGGAGAGGCAGATGGAATTGGTGGTGTAGGGGTAAAATCCGTA-GA-3′) (Fontanot et al., 2014) in the biochip system.

The DNA sequences retrieved from GenBank using the following accession numbers: HM007568.1, DQ174142.1, DQ174141.1 for *Campylobacter jejuni*, HM007569.1, AB542728.1, JX912505.1 for *C. coli*, GQ167657.1, AF550634.1 for *C. lari,* and DQ174157.1, AF497805.1, GQ167658.1 for *C. upsaliensis*, AY277975.1 for *Helicobacter ganmani* CCUG 43527, and AY277974.1 for *H. ganmani* CCUG 43526 were analyzed.

The probes were tested in silico using Blast (http://blast.ncbi.nlm.gov/Blast.cgi), before being used in bio-chip construction. The CampyDet probe, after the labelling of the 5′ end with digoxigenin (Dig-CampyDet probe), was tested using the dot blot technique (Fontanot et al., 2014) on the synthetic ssDNA sequence, complementary to the CampyDet probe, to evaluate the DNA probe sensitivity and to establish the optimal concentration of the labelled probe for use in the experiments. The DNA extracted from the reference strains listed in Table 1 according to Manzano et al. (2003) was also used in the dot blot procedure, to test the specificity of the probes. Moreover, DNA from *Helicobacter suis* 19735 (DSM), *Arcobacter cryaerophilus* 7289 (DSM) were used.

2.4. Silanization of the glass slides and capture-probe binding

As support of the biochip, microscope cover glasses ($28 \times 19\ mm^2$) were used. For silanization the protocol described by Marcello et al. (2013) was followed with some modifications. The cover glasses were treated with 10% NaOH (2.5 mM, Sigma, Italy) at room temperature for 1 h, rinsed with deionized water and treated with 0.1 N HCl for 15 min. After a washing step with deionized water, the glass slides were rinsed in acetone and dried at 50 °C for a few minutes, and immersed in a 0.5% APTES (3 aminopropyltriethoxysilane) (Fluka, Milan, Italy) solution in deionized water for 30 min at room temperature. Slides were then rinsed three times in deionized water followed by 10 min washing under shaking, dried at 160 °C for 1 h and cooled at room temperature for 30 min. After modification at 5′ end with an amino group instead of the digoxigenin (amino-capture-probe), 1 μL of the amino-probe at 100 ng/μL, in triplicate, were drop off on each glass slide and incubated at 4 °C overnight to bound to microscope cover glass surface.

2.5. Labelling of the detection probe by Alexa Fluor® 430

For the first time the fluorophore Alexa Fluor® 430 (Invitrogen, Monza, Italy) that exhibits the absorption between 400 and 450 nm and fluorescence emission beyond 500 nm, was bound to a DNA molecule and subsequently used in a biochip detection system.

250 μg of Alexa Fluor® 430 was mixed with 14 μL of DMSO (Dimethyl sulfoxide)(Sigma, Milan, Italy), then 7 μL of nuclease free water, 75 μL of sodium tetraboroidrate 0.1 M (pH 8.5) and 4 μL of the CampyDet probe at 25 μg/μL, with an amino group at the 5′ end, were added. The tube was incubated overnight at room temperature under agitation for 2 h. After incubation 10 μL NaCl 3 M and 250 μL cold absolute ethanol, were mixed in the tube and incubated at −20 °C for 30 min. The tube was then centrifuged at $12{,}000 \times g$ for 30 min. The supernatant was discarded and the pellet washed two times with cold ethanol at 70%, dried for few min, resuspended in 200 μL of 50% formamide was loaded into an agarose gel at 2% for purification. The band containing the labelled probe (Alexa-CampyDet) was cut out from the gel, soaked in sterile deionized water overnight at 4 °C. The eluted Alexa-CampyDet probe was collected in a new tube and maintained at −20 °C till utilization.

2.6. Fluorescence optical image acquisition and processing

For all the tests the fluorescence signal was acquired using a high sensitive camera (Hamamatsu Orca C8484-03G02) integrated with a microscope objective. The images were acquired with the gain set to 1 and with an integration time of 30 s. The image digitalization was set at 12 bit with a grey scale ranging from 0 to 4095. The image processing was performed with the free software ImageJ (http://rsb.info.nih.gov/ij/, 1997–2009). A process of despeckle to eliminate scattering luminous spots have been applied to all the images as well as a threshold adjustment to identify the emitting areas. Mean intensity of the identified area was measured and background subtracted, analysis and plotting was performed in R (R Development Core Team, Computing R. F. f. S., Ed., Vienna, Austria, 2010).

2.7. Construction of the bio-sensor sensitivity curve

The characterized DNA probes were then used for the biosensor construction.

A bottom-emission small molecule-based OLED, which was optimized to obtain a deep-blue (DB) colour emission with a peak wavelength of 434 nm (DB-OLED-Marcello), was used to excite the fluorescence of the commercial dye Alexa Fluor® 430 (Invitrogen, Monza, Italy), with the absorption peak located at 434 nm and the emission peak located at 541 nm (Panchuk-Voloshina et al., 1999). An in-depth physical description and characterization of the patented OLED, adopted in this bio-sensor, using the fluorescent molecule, a-NPD [N, N′-diphenyl-N, N′-bis (1-naphthylphenyl)-1, 1′-biphenyl-4, 4′-diamine] as an emitter, has been reported by Marcello et al. (2013).

One hundred-micrometre-thick silanised microscope cover glasses ($28 \times 19\ mm^2$) were used as support of the bio-probes employed in the biochip. The DNA capture probes after a modification at the 5′ end by adding an amino group instead of the digoxigenin (amino-capture-probe), were deposited on the silanised glasses. A 0.5 μL drop of the amino-capture-probe at 100 ng/μL was deposited on each glass slide and incubated at 4 °C overnight to bind to the glass surface.

The glass slides with the bound amino-capture probe were washed twice in deionised water prior to utilization. Then, 0.5 μL of the DNA samples and 0.5 μl of the Alexa-CampyDet probe (CampyDet probe labelled at the 5′ end with the Alexa Fluor® 430 dye) were spotted on each slide in a ratio 1:4 after 5 min of denaturation at 95 °C. A sensitivity curve was determined using different concentrations of DNA (25 ng/μL, 12.5 ng/μL, 6.25 ng/μL, 3.12 ng/μL, 1.50 ng/μL, and 0.75 ng/μL) from *C. jejuni* subsp. *jejuni* ATCC 49943, and various concentrations (100 ng/μL, 50 ng/μL, 25 ng/μL, 12.5 ng/μL, 6.25 ng/μL, 3.12 ng/μL, and 1.5 ng/μL) of the Alexa-CampyDet probe. The microscope cover glasses were incubated at 63 °C for 1.5 h in a sterile petri dish to prevent evaporation, and they were washed twice in sterile deionised water to eliminate the unbound DNA and the unbound Alexa-CampyDet probe.

Finally, the glass slides were assembled on a rectangular DB-OLED together with a high-pass optical excitation filter with a high extinction at the wavelength corresponding to the fluorophore emission (transmission $(T) < 10^{-5}$) and a high transmission in the excitation spectral region. A second bandpass filter centred on the fluorophore wavelength emission was used before the signal capture camera. The DB-OLED was used at 7.0 V with a total optical energy density of 85 mW/cm^2 (Banerjee et al., 2010). The fluorescence signal was acquired with a high sensitivity CCD camera, acquiring an image with a 12- bit digitalisation. The CCD gain was set at the maximum value and an integration time of 30 s was used for image acquisition. For the analysis, the background of each

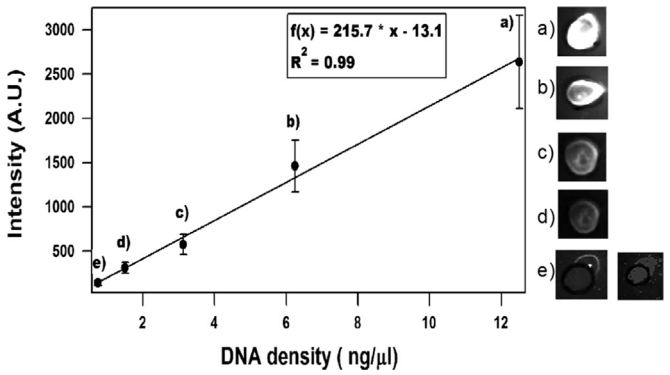

Fig. 1. Sensitivity curve obtained using different DNA concentrations. The mean number of counts (ranging from 0 to 4095) of the fluorescent signals, recorded with a 12 bit digitalization, is reported as a function of DNA concentration. Different images of the obtained fluorescence signals are shown: (a) DNA concentration of 12.5 ng/ml; (b) DNA concentration of 6.25 ng/ml; (c) DNA concentration of 3.12 ng/ml; (d) DNA concentration of 1.5 ng/ml; and (e) DNA concentration of 0.75 ng/ml. For clarity at the last image is associated its thresholded image. The DNA concentration of 25 ng/ml produced a signal in saturation not reported in the sensitivity curve. The measured fluorescence signals present a linear behaviour as a fuction of DNA concentration (R^2=0.99).

image was subtracted; a de-speckle algorithm was used, and the images so obtained were thresholded to automatically identify and measure the circular spots. To calculate the sensitivity curve, the mean value of the spot was considered and the results are reported in Fig.1.

2.8. Real samples analyses by classical plate method, molecular methods and the OLED biochip

After the determination of the sensitivity curve, an experiment using real poultry meat samples was performed. Seven poultry samples were collected from local markets and analyzed for the presence of *Campylobacter* spp. according to the official method ISO 10272-1B: 2006 (Voedsel en Warent Autoriteit, 2010) and by direct plating on the selective modified Charcoal–Cefoperazone–Deoxycholate Agar (mCCDA) (Oxoid, Milan, Italy). Samples were also evaluated for the enumeration of mesophilic aerobic microorganisms and *Enterobacteriaceae*.

Twenty-five grams of skin from each poultry sample was transferred to a filter sterile Stomacher bag (PBI, Milan, Italy); 100 mL of saline-peptone water was added (8 g/L NaCl, 1 g/L bacteriological peptone, Oxoid, Milan, Italy), and the contents were mixed for 1.5 min. Aliquots of 0.1 mL were used to obtain the mesophilic aerobic count on Plate Count Agar (PCA, Oxoid) and to count *Campylobacter* spp. on mCCDA, whereas aliquots of 1 mL were used for the enumeration of *Enterobacteriaceae* on Violet Red Bile Glucose (VRBG) agar (37 °C for 24 h). DNA was extracted from 10 mL saline-peptone water and used for the homogenization of the samples, purified as described by Manzano et al. (2003); these samples were used in the molecular methods reported and in the OLED biochip system proposed in this work. Then, 0.5 µL of the DNA extracted from the physiological solutions used to homogenize each chicken sample, and standardised at 25 ng/µL, was mixed with 0.5 µL of the Alexa-CampyDet probe (Alexa Fluor® 430 labelled) at 100 ng/µL and spotted (in triplicate) onto the glass slides holding the amino-capture probe. The glass slides were placed on the OLED, and the fluorescence was measured by the CCD camera using the acquisition parameters described above. The fluorescence value reported for each sample was obtained as an average value of three measurements. DNA was also extracted from the Preston enrichment broths after 48 h, purified and used in dot blot and PCR assays.

The colonies grown on mCCDA and suspected to be *Campylobacter* based on morphology were isolated and tested for motility, oxidase and catalase activity and growth at 25 °C, Gram stained and assayed by PCR using the protocol proposed by Fontanot et al. (2014). Amplicons obtained by PCR were sent to Eurofins MWG Operon (Eurofins MWG Operon, Ebersberg, Germany) for sequencing.

3. Results

3.1. Specificity and sensitivity of the DNA probes

The sensitivity of 20 pmol/mL digoxigenin-labelled capture probe was determined by dot blot to be 1 ng/µL using the synthetic ssDNA oligonucleotide sequence complementary to the probe as the target, and 25 ng/µL using the genomic DNAs extracted from the *Campylobacter* reference strains reported in Table 1 as the target. The probe showed high specificity under the conditions proposed in the described protocol, annealing only to the *Campylobacter* species used as reference strains. The sensitivity of the 55-base Dig-CampyDet probe (20 pmol/ml) was determined by dot blot to be 1 ng/µL using the ssDNA complementary sequence and 25 ng/mL using pure culture reference strains as the target DNA.

The sensitivity curve obtained using the OLED biochip is reported in Fig. 1. The serial dilutions of the tested DNA are plotted against the number of counts measured in the luminous spots (count range between 0 and 4095). The results obtained by the biochip system using various concentrations of DNA from *Campylobacter* pure culture show an excellent linearity (R^2=0.99). The linear regression interpolation parameters are also reported in Fig. 1.

As observed from Fig. 1, the OLED biochip, which included both the amino-capture-probe and the Alexa-CampyDet probe, showed a sensitivity of at least 0.37 ng/µL, as obtained by extrapolating the sensitivity curve, which is almost two orders of magnitude higher than the sensitivity obtained with the dot blot method (25 ng/µL). Moreover, the efficacy of the method in preventing false negative results was evaluated using various negative samples: (a) no capture probe (omission of the amino-capture probe); (b) no DNA (omission of the DNA sample); and (c) no Alexa (omission of the Alexa-CampyDet probe). These control experiments confirmed the specificity of the test (images not shown). The images obtained (in triplicate) using the DNA of *Campylobacter* (a positive sample) at a concentration of 6.25 ng/µL show high level of fluorescence, whereas the images obtained using DNA of *Enterobacter cloacae* (a negative sample previously tested by dot blot as well) at the same concentration do not show any fluorescence signal, as reported in Fig. 2.

3.2. Real poultry meat samples analyses results

The results of the classical plate count, PCR, dot blot and OLED biochip tests are reported in Table 2. *Campylobacter* spp. were detected in four of the seven chicken samples analyzed by direct plating onto mCCDA and varied from 15 CFU/g (colony forming units per gram) to 3.6×10^3 CFU/g, whereas three samples were below the detection limit of the method (<5 CFU/g).

Samples 1, 2, 4 and 7, which exibited growth of *Campylobacter* on the selective medium mCCDA both, after direct plating from the homogenization solution, and after the enrichment step in Preston broth (isolates), were also positive for *Campylobacter* according to PCR, dot blot and the OLED biochip analysis.

In fact, isolates from mCCDA were confirmed to be *Campylobacter* spp. by motility test, Gram staining, oxidase activity, catalase activity and growth at 25 °C. The DNA sequences corresponding to the amplicons produced by PCR, obtained from Eurofins MWG Operon centre, matched 100% the *Campylobacter* sequences retrieved from GenBank using Blast (http://blast.ncbi.

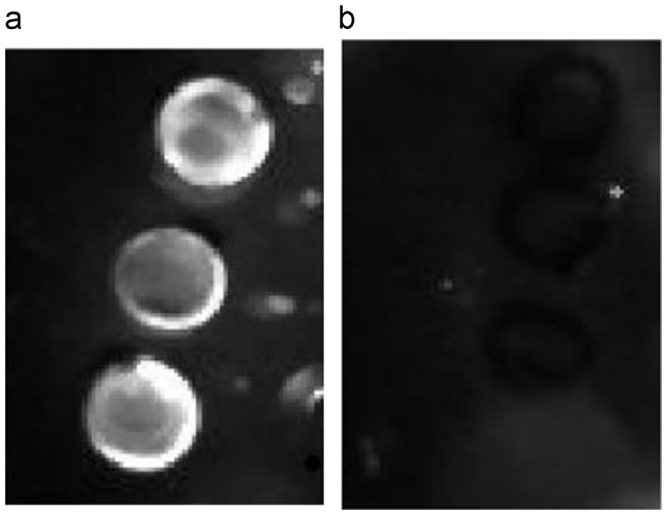

Fig. 2. Fluorescence images obtained hybridizing at 63 °C 6.25 ng/ml DNA (in triplicate) extracted from pure cultures of two different bacteria. The fluorescent signal present for *Campylobacter jejuni* (a) indicates a positive result (around 1500 AU), whereas the absence of fluorescence showed for *Enterobacter cloacae* (b) indicates a negative result (< 10 AU). In both the measures of the two images the background was subtracted.

Table 2
Data of the microbial evaluation of 7 chicken samples using plate count method (PCA, VRBG and mCCDA) (values expressed in colony forming units (CFU)/g); PCR, dot blot, and the OLED bio-chip (results expressed as presence (+) or absence (−)). OLED was used on samples from physiological solution immediatly after homogenization, whereas PCR and dot blot on DNA extracted from Preston broth after 48 h enrichment.

Sample	mCCDA[a]	PCR[b]	PCA[c]	VRBG[d]	Preston broth isolates[e]	OLED[f]	Dot blot
1	1.5×10^1	+	4.0×10^4	5.3×10^3	+	+	+
2	1.5×10^1	+	6.5×10^7	6.0×10^5	+	+	+
3	< 5	−	3.6×10^6	1.8×10^3	−	−	−
4	3.6×10^3	+	3.1×10^8	7.0×10^5	+	+	+
5	< 5	−	3.7×10^7	3.9×10^5	−	−	−
6	< 5	−	5.9×10^8	3.3×10^7	−	−	−
7	1.6×10^3	+	3.0×10^4	2.7×10^3	+	+	+

[a] Modified Charcoal–Cefoperazone–Deoxycholate Agar.
[b] PCR was performed according to the protocol described by Fontanot et al. (2014).
[c] Plate Count Agar.
[d] Violet Red Bile Glucose Agar.
[e] Positivity was assigned when *Campylobacter* colonies were present onto the selective media (isolates), and when the blue spot due to the annealing of the specific probe was obtained.
[f] Positivity was assigned when AU (Arbitrary Units) values were above 500 AU.

nlm.nih.gov/Blast.cgi), thus confirming the identification of *Campylobacter* obtained by PCR (Table 2). The blue spots obtained for the samples 1, 2, 4 and 7 through the hybridization of the specific digoxigenin-labelled probe used in the dot blot with the DNAs extracted from the Preston broths confirmed the presence of *Campylobacter*.

In addition, the OLED biochip analysis confirmed the positivity of the samples 1, 2, 4 and 7. In fact, the luminosity values obtained in the positive samples are consistent with the results obtained for the sensitivity curve (Fig. 1). The images of two samples, i.e., the positive chicken meat sample number 2, and the negative chicken meat sample number 3, which were analyzed using the DNA extracted from the physiological solutions, thus without any enrichment step in broths, are reported in Fig. 3.

A cut-off value of luminescence count was set at approximately 500 AU, which corresponds to a negative meat sample in all of the

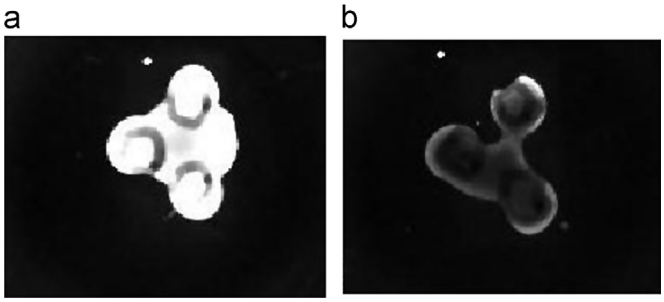

Fig. 3. Fluorescence images (in triplicate) obtained with DNA extracted from physiological solutions used for homogenizing two chicken meat samples (without enrichment process), after the background subtraction. Sample 2 (a) showed an intense fluorescence signal indicating the presence of *Campylobacter* (> 3400 AU), and thus considered positive. Sample 3 (b) showed a low fluorescent signal, lower than the cut-off limit (< 500 AU), indicating the absence of *Campylobacter* and thus considered negative. These results were confirmed by PCR, dot blot and plate count method.

other tests (the ISO 10272-1B: 2006 (no colony growth onto selective agar plates), PCR (no amplicons obtained) and dot blot (no blue spots), to allow the discrimination between positive and negative samples. For this reason, samples below this value (3, 5 and 6) were considered negative. The values between 0 and 500 AU, which were obtained for some negative real meat samples, could be due to the presence of dead *Campylobacter* cells, which are able to anneal the specific Alexa-CampyDet probe and produce a weak luminescence that is detectable due to the high sensitivity of the system. The mesophilic aerobes varied from 3.0×10^4 to 5.9×10^8 CFU (colony forming units)/g, and those of *Enterobacteriaceae* varied from 1.8×10^3 to 3.3×10^7 CFU/g. The microbial contamination evaluated on PCA and VRBG indicates that the presence of *Campylobacter* is not affected by the hygienic level of the samples; in fact, it can be present both in low and high levels of sample contamination. A systematic control for the presence of *Campylobacter* spp. should also be performed in meat companies that have a satisfactory level of hygiene during meat samples processing.

4. Discussion

The detection and identification of *Campylobacter* spp.-contaminating poultry meat samples is usually carried out by culturing techniques that are laborious and time consuming. The utilization of molecular methods such as PCR and dot blots allow for the faster detection of *Campylobacter* spp. in food samples, because these methods can be applied to DNA extracted from the Preston enrichment broth without requiring cell growth (Silva et al., 2011). Moreover, molecular techniques are able to detect the VBNC (Oliver, 2005) forms of *Campylobacter* spp. that often do not grow on selective media due to the stressing conditions of food, although they are present in food. Comparing the time required to obtain results, we can assert that the molecular methods used are convenient in comparison with plate count methods because they are able to give results within 48–72 h, whereas classical microbiological methods require approximately one week. Due to its sensitivity, the OLED biochip proposed is even more rapid, and takes only 24 h.

Moreover, the OLED biochip was able to detect *Campylobacter* using the DNA that was extracted directly from the physiological solutions used for the homogenization of the chicken meat samples. Thus, it allows for the first time, for *Campylobacter* spp. to be detected without any prior enrichment step in Preston broth, a step that is necessary for obtaining sensitive results in the current commercial ELISA tests and qPCR assays. The OLED biochip

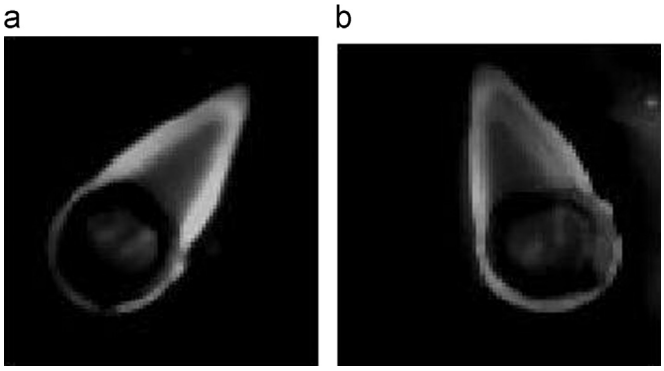

Fig. 4. Comparison of the fluorescent signal obtained by the same sample of DNA of *Campylobacter jejuni* at a concentration of 6.25 ng/ml measured just after the sample preparation (a) and one month later (b), after background subtraction. The two samples have been measured with two different OLEDs at the same optical power density (85 mW/cm^2) giving the following results: (a) 1450 AU and (b) 1380 AU.

reached a sensitivity of 0.37 ng/μL DNA, which was approximately 20-fold higher than the sensitivity obtained with dot blot assay (25 ng/μL), when performed under the same conditions using DNA extracted from the reference strains listed in Table 1.

The detection of *Campylobacter* in a chicken sample by dot blot requires a 48-h enrichment step in Preston broth to allow *Campylobacter* cells to multiply and to reach a number detectable by this method, whose sensitivity is 20-fold lower than the OLED biochip sensitivity. The OLED biochip system is also highly specific as demonstrated by the analysis made using the DNA probes on the various microorganisms reported in Table 1, considering that only *Campylobacter* DNAs gave positive results using the two DNA probes designed.

The ability of the biochip to measure real meat samples has also been demonstrated by the absolute correlation obtained with the standard methods used to analyze the poultry meat samples to detect *Campylobacter* spp., as shown in Table 2, when applying a minimum AU value (cut-off value) of approximately 500 counts. Based on this assumption, the same samples positive by the OLED biochip were also positive by the other methods used, including the direct plate count on mCCDA, which shows a minimum level of detection of 5 CFU/g. This correlation among the different methods adopted for the analysis validated the obtained results. Another very important consideration is that this method of analysis preserves the fluorophore functionality after the first analysis. The low optical energy density used to excite the fluorescence (approximately 85 mW/cm^2) does not produce a photobleaching effect on the dyes, as is common in other optical tests that uses high optical energy density from unoptimised sources (laser or high power LED). Due to the long stability time of the used and undamaged fluorophore, the proposed OLED biochip is a non-destructive assay. If stored at 4 °C in a dark environment, the biochip can be reused after months, giving nearly the same fluorescence values when measured immediately after its preparation, and excited with the same optical energy density. Two fluorescence images of the same sample (*Campylobacter* DNA at 6.25 ng/μl), measured at the first sample analyses and one month later, after storage at 4 °C in a dark place, are shown in Fig. 4. The measured fluorescence taken after one month, with a different OLED source but at the same optical energy density, shows a difference of less than 5% compared with the value acquired immediately after the preparation. The stable biochip allows the storage of the samples analyzed for possible successive checks thereby confirming the robustness of the proposed system.

5. Conclusions

The OLED-based DNA biochip proposed in this paper is 20-fold more sensitive than the classical plate methods and molecular methods previously developped; it is easy to use, and stable, as samples can be re-analyzed after one month conservation and no variations in the measures are obtained. Next steps will be the complete authomatization of the extraction of DNA for the inclusion in a completely automated diagnostic system.

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