A Highly Sensitive Detection of *Campylobacter spp.* in Chicken Meat using Silica Nanoparticle Enhanced Dot Blot DNA Biosensor

Priya Vizzini[a,b], Marisa Manzano[b], Carole Farre[c], Thierry Meylheuc[a], Carole Chaix[c], Nalini Ramarao[a,*], Jasmina Vidic[a,*]

[a]Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France.

[b]Dipartimento di Scienze AgroAlimentari, Ambientali e Animali, Università di Udine, Italy.

[c]Institut des Sciences Analytiques, UMR 5280, CNRS-UCBL, Université de Lyon, Lyon, France.

[*]Corresponding authors

E-mail address: jasmina.vidic@inrae.fr ; nalini.ramarao@inrae.fr

Phone: + 33134652737

Abstract

Paper-based DNA biosensors are powerful tools in point-of-care diagnostics since they are affordable, portable, user-friendly, rapid and robust. However, their sensitivity is not always as high as required to enable DNA quantification. To improve the response of standard dot blots, we have applied a new enhancement strategy that increases the sensitivity of assays based on the use of biotinylated silica-nanoparticles (biotin-Si-NPs). After immobilization of a genomic *Campylobacter* DNA onto a paper membrane, and addition of a biotinylated-DNA detection probe, the hybridization was evidenced using streptavidin-conjugated to horseradish peroxidase (HRP) in the presence of luminol and H_2O_2. Replacement of the single biotin by the biotin-Si-NPs boosted in average a 30 fold the chemiluminescent read-out of the biosensor. The characterization of biotin-Si-NPs into a paper with immobilized DNA was done using a scanning electron microscope. A limit of detection of 3 pg/µL of DNA, similar to the available qPCR kits, is achieved but being cheaper, easier and avoiding the inhibition of DNA polymerase by molecules from the food matrices. We demonstrated that the new dot blot coupled to biotin-Si-NPs successfully detected *Campylobacter* from naturally contaminated chicken meat, without a need for PCR step. Hence, such enhanced dot blot paves the path to the development of a portable and multiplex paper based platform for point-of-care screening of chicken carcasses for *Campylobacter*.

Keywords: *Campylobacter,* DNA dot blot, Si-nanoparticles, Food safety, Multiplex bacterial detection.

1. Introduction

Campylobacter spp. are the most common bacterial cause of human gastroenteritis in the world. The World Health Organization (WHO) estimates that 550 million people fall ill every year, of which 220 million are children (WHO 2020). This zoonosis is transmitted mostly through the exposure to under-cooked poultry products (50-80% cases), and in a minor way, to contaminated milk, vegetables and water (ECDC 2018; Hermans et al. 2012; Humphrey et al. 2007). Campylobacteriosis in humans is usually a self-limiting condition involving bloody diarrhea, abdominal cramping, nausea and fever, which can last up to two weeks. In 1 % of cases, campylobacteriosis goes on to the Guillain-Barré syndrome, a severe autoimmune disease that leads to death in 2–12 % of sufferers, depending on their age (Scallan Walter et al. 2020). The overall economic burden of campylobacteriosis was estimated to up to EUR 3 billion/year in EU (ECDC 2018), and between US$ 1.2 and 4 billion/year for the US (Batz et al. 2014). To prevent the entry of *Campylobacter* contaminated broilers into the market, the European Commission adopted a process hygiene criterion (EU 2017/1495) with the critical limit of 1000 cfu/g of boiler meat or skin and enforced systematic screening of broiler carcasses for *Campylobacter* spp. (EU No 2017/625).

Identification and quantification of *Campylobacter* spp. rely on conventional, culture-based methods, and bacterial phenotypical characterization (Vizzini et al. 2019). *Campylobacter* is highly infectious, with reported infective doses as low as 500 cells (Black et al. 1988). Because such low amounts of *Campylobacter* are difficult to detect, food samples have to be incubated in an enrichment broth for 24–48 h before culturing. Culturing of *Campylobacter* is slow, requiring 48–72 h, and involves specialized medium and chambers for microaerophilic growth. *Campylobacter* is usually present in low titers in a food sample when compared to other bacteria, like coliforms and Enterobacteriaceae, making it difficult to

isolate. The official ISO 10272-1:2006 method for *Campylobacter* detection may provide false negative results because of the tendency of *Campylobacter* to die erratically during handling. Furthermore, *Campylobacter* can enter in a viable but not culturable (VBNC) status in food matrices, which makes its detection based on culturing impossible (Vidic et al. 2017; Vizzini et al. 2019). Identification of *Campylobacter* by the optical microscope is not easy either as bacteria can change their spiral distinctive shape into spherical or coccoid. Moreover, the current problem of official methods concerns the use of an insufficiently selective medium, and emergence of bacterial resistance against the antibiotic added to the enrichment broth.

Culture-independent molecular methods, such as PCR, real-time PCR and qPCR, are used as alternatives to colony growth (De Boer et al. 2015; Fontanot et al. 2014; Gosselin-Théberge et al. 2016; Liu et al. 2017; Ricke et al. 2019). However, PCR-based methods may also provide false-negative responses because of the sensitivity of DNA polymerase to inhibitors present in food matrices and enrichment broths (Schrader et al. 2012; Vidic et al. 2019). Next generation sequencing (NGS) which enables for the sequencing of the entire bacterial genome in a relatively short time stars to be used as a tool for identification of the infectious bacteria. However, NGS can hardly be routinely used at farms and slaughterhouse because it requires special equipment and highly trained person to interpret data (Gosiewski et al. 2017).

Biosensors for detection of *Campylobacter* show marked advantages over traditional methods in terms of rapidity, facility of utilization and cost-effectiveness (Manzano et al. 2015; Masdor et al. 2016; Morant-Miñana and Elizalde 2015; Vidic et al. 2017; Vidic et al. 2019; Vizzini et al. 2019; Yang et al. 2013). However, today no commercial biosensor is available to detect *Campylobacter* in food matrices, mainly because of the difficulty to reach high sensitivity.

Due to significant improvement in biosensor technology over the last two decades, applications of paper-based sensors in pathogen detection are increasing. Such devices seem to meet ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free and Deliverable) recommended by the WHO for point-of-care diagnostic. Paper as a substrate has the advantages to be inexpensive, lightweight, and easy to enable multiplex analysis (Dincer et al. 2019). Various paper-based methods to detect foodborne pathogens have been reported including enzymatic-, immuno- and DNA/RNA- tests (Jokerst et al. 2012; Morales-Narváez et al. 2015; Russell et al. 2017). Tests that target nucleic acids are among the most accurate and specific although they are usually associated with a PCR step, including isothermal amplification, to allow for a detection of pathogens in low titers (Trinh et al. 2020; Vidic et al. 2019).

Here, we coupled functionalized silica nanoparticles (Si-NPs) to a paper based DNA dot blot test to enable sensitive *Campylobacter* nucleic acid detection without a pre-amplification step. A highly specific DNA probe that recognizes the 16S rRNA gene of most prevalent *Campylobacter* spp. causing infections (*C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis*) was used in the test. Si-NPs decorated with biotin (biotin-Si-NPs) were employed to enhance strepatavidin–HRP chemiluminescent signal read-out. Our study illustrates the proof-of-concept that the collective effect of biotin-Si-NPs could become an effective mean for increasing the sensitivity of the cost-effective, specific and easy-to-perform dot blot assay.

2. Material and Methods

2.1 Materials and reagents

Streptavine-HRP, Proclin, acetonitrile, 1,8-Diazabicyclo[5.4.0]undéc-7-ène (DBU), Controlled pore glass (120-200 Mesh, CPG-3000 Å) were purchased from Sigma-Aldrich (Saint

Quentin Fallavier, France). Fluorescent rhodamine B silica NPs (Si-NPs, diameter~50 nm) were supplied by Nano-H (Saint Quentin Fallavier, France). DNA phosphoramidite synthons and all DNA-synthesis reagents were purchased from Glen Research (Sterling, Virginia, USA). Phosphate buffered saline (PBS) was purchased from Dominique Dutscher (Brumath, France). Amersham Hybond-N+ nylon and Amercham Hybon-XL nitrocellulose membaranes were purchased from ThermoFisher (Illkirch, France).

All bacterial media and supplements used were from Oxoid (Italy), except for Violet red Bile glucose (VRBG) agar and Coli ID medium that were purchased from Biomerieux (Italy). Triton, SDS, NaCl, trizma-base, phenol, chloroform, bacteriological peptone and isoamyl alcohol were purchased from Sigma (Milan, Italy). A 36-mer oligonucleotide related to the 16S gene encoding for ribosomal *Campylobacter* RNA of *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* (base location: 72-108) was use as a recognition element (probe named CampyP3). CampyP3 was labeled in at 5' with biotin for chemiluminescent dot blot assay development. The probe was tested *in silico* by the OligoAnalyzer3.1 (https://eu.idtdna.com/calc/analyzer), the Amplifix software, Fast PCR 6.1 and Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ssDNA sequence complementary to CampyP3 probe, named CP3, and two non-specific ssDNA sequences, named PR and PE, respectively were designed to study selectivity of the sensor through their hybridization with the probe. PR was design by mismatching positions of nucleic acids of the CP3 sequence, while PE corresponded to the sequence of *E. coli* that shows some similarities to *Campylobacter* (base location: 338-376 for the sequence of *E. coli* corresponding to assession number 527445.1). All designed sequences are presented in Table 1. They were provided by Sigma-Aldrich (Saint Quentin Fallavier, France) as lyophilized powers. All solutions were prepared using Milli-Q water.

2.2 *Bacterial strains*

Bacteria strains used in this study are listed in the Table 2. *Campylobacter* strains were grown under microaerophilic conditions (5% O_2, 10 % CO_2 and 80% N_2) in anaerobic gas jars. Microaerophilic conditions were generated with a Sachet Oxoid™ CampyGen™ 2.5 L (Oxoid, Italy) at 37°C for 48h. *Campylobacter* isolates cultured on Columbia blood agar plates (both pure strains and isolates from chicken samples) were verified by Gram straining and optical microscope observations for cell morphology and motility (Brucella broth, Thermofisher, Italy) after oxidase and catalase tests.

All negative control strains were grown on the specific media at optimal conditions and subjected to the same tests carried out for *Campylobacter* before DNA extractions.

2.3 Sample collection, plate count enumeration and selective isolation

Five chickens were purchased from local supermarkets in Italy. 10 g of chicken skin were transferred into a filter-sterile stomacher bag with 40 mL saline-peptone water (8 g/L NaCl, 1 g/L bacteriological peptone) and homogenized in a Stomacher (PBI, Milan, Italy) for 90 s. Aliquots of 0.1 mL were used for the mesophilic aerobic count on Triptone Soya Agar at 30°C for 48 h, and yeast/molds on 48 h for Agar Malt tetracycline at 30°C for 48 h. Aliquots of 1 mL were used for enumeration of Enterobacteriaceae on VRBG agar (37°C for 24 h), and coliforms and *E. coli* on Coli ID medium (37°C for 24 h). *Campylobacter* detection was performed according to the conventional method ISO 10272-1:2006. For selective bacterial isolation, 10 µL of the Bolton broth were streaked onto Skirrow agar plates, and mCCDA (modified charcoal-cefoperazone-deoxycholade) plates and incubated at 41.5 °C for 48h, under microaerophilic conditions. One colony was selected from mCCDA and streaked on two plates of Columbia blood agar. One plate was incubated at 41.5 °C for 48 h in aerobic condition and

one plate at 25 °C for 48 h in microphilic condition. Growth on Skirrow served as a confirmation to proceed with the identification steps.

2.4 DNA extraction from pure cultures and enrichment broths

DNA was extracted from pure strains and isolates from chicken samples according to (Manzano et al. 2015). 2 mL from the bacterial culture in Bolton enrichment broth were harvested through centrifugation at 13,000 g at 4 °C for 10 min. Bacterial pellets were washed three times with PBS, and subjected to extraction as previously reported (Manzano et al. 2015). Extracted DNAs were rehydrated using 50 µL of sterile distilled water and treated with RNase enzyme at 37°C for 1 h. Finally, DNA was quantified using Nanodrop™ 2000C (ThermoFisher Scientific, Italy). Concentration of DNA was adjusted to 100 ng/mL concentration using sterile distilled water.

2.5 Sample immobilization and detection procedure

Prior to immobilization, extracted DNA samples were denatured at 95 °C for 10 min, put immediately on ice and 1 µL spotted on the positively charged nylon membrane (Amersham Hybon™-XL, GE Healthcare, France). Spotted membrane was exposed to UV at 254 nm for 10 min to fix DNA. The membrane was soaked in pre-wormed hybridization buffer (0.5 M Na_2HPO_4, 0.5 M NaH_2PO_4, 10 mM EDTA, 1 % SDS, pH 7.5) at 65°C for 30 min under gentle shaking. 4 ng/µL of the denatured biotin labeled CampyP3 probe (100 ng/µL) were added to hybridization buffer and left overnight at 65°C under gentile shaking to allow for hybridization.

The membrane was washed twice with 0.1 SDS, 300 mM SSC (Saline sodium citrate, Meraudex, France) for 5 min and with 75 mM SSC for 15 min. The membrane was transferred in the blocking buffer (0.1 % Tween, 0.1 % BSA, 0.03% Proclin, PBS, pH 7.4) at room temperature for 15 min to saturate the surface. The membrane was finally incubated with the

blocking solution containing 0.7 µM streptavidin-HRP for 15 min at room temperature. After washing with 0.1 % SDS, 150 mM SSC at room temperature for 5 min, the membrane was transferred to a new petri dish and incubated with 10^6 biotin-Si-NPs/mL, PBS, pH 7.4 under gentile shaking at room temperature, for 30 min. A 0.7 µM streptavidin-HRP in blocking solution was added after washing and incubated for 30 min with shaking. The signal was then revealed using the enhanced chemiluminescent substrate for detection HPR (Thermo Scientific, France). The membrane was removed from the solution and observed under a ChemiDoc MP imaging system (Biorad, France). Detection signals were quantified using Image Lab™ software (Biorad). The normalized value of a spot intensity was calculated by $(PI_0 - PI_n)/PI_0$, where PI_0 and PI_n represent the pixel intensity obtain for detection of 0.1 ng/µL CP3 probe (standard) and the experimental sample, respectively.

2.6 Scanning electron microscopy

Samples were mounted on aluminum stubs (50 mm diameter) with carbon adhesive discs (Agar Scientific, Oxford Instruments SAS, Gomez-la-Ville, France) and visualized by field emission gun scanning electron microscopy (SEM FEG) as secondary and backscattered electrons images (5 kV) under high vacuum conditions with a Hitachi SU5000 instrument (Milexia, Verrières-le-Buisson, France). Samples preparation and scanning Electron Microscopy analyses were performed at the Microscopy and Imaging Platform MIMA2 (INRAE, Jouy-en-Josas, France).

2.7 Si-NP synthesis, labeling and quantification

Si-NPs was functionalized by an innovative solid-phase synthesis technology which enables functionalization of nanosized particles with DNA fragments, as reported previously (Bonnet et al. 2018; De Crozals et al. 2012). Briefly, the nanoparticle immobilization onto

controlled pore glass (CPG) allows a very high functionalization with a modified oligonucleotidic based linker. The linker (sequence: dT10-PEG2-dT10) was synthetized using an applied Biosystems 394 RNA/DNA synthesizer (Applied Biosystems). After grafting nanoparticles on CPG support, they were functionalized by automated synthesis using the phosphoramidite chemistry. First, the linker, that allows a better accessibility of the functions of interest, was synthesized. Second, the biotin group was incorporated. We controlled the biotin incorporation to reach a 10% coupling yield. To do it, diluted solutions of biotin phosphoramidite (10 mM) and tetrazole in acetonitrile (45 mM) were used and the coupling time was reduced to 10 s. The biotin incorporation was monitored at 498 nm using dimethoxytrityl quantification by UV-visible spectrophotometer. Third, biotin-Si-NPs were released from CPG by incubating the support in 1 mL of 0.1% (m/v) DBU in water-acetonitrile 1/1 (v/v). The DBU solution was stirred in a thermomixer at 22°C during 1 h before recovering. A fresh DBU solution was added to the CPG suspension every hour. The release kinetics was followed by quantifying NPs concentration in each DBU solution with UV-visible measurement at 560 nm. Released nanoparticles were washed with milli-Q water (1x 4 mL then 3 x 2 mL) and concentrated on 30 K Amicon Ultra filter (5000 g, 10 min). Finally, the amount of dT10-PEG2-dT10-10% Biotin strands per NP was estimated using Varian Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies) using a quartz cuvette of 1 cm path length.

The amount of strands per NP was estimated using Varian Cary 100 Bio UV-visible spectrophotometer (Agilent Technologies) using a quartz cuvette of 1 cm path length. The amount of linker grafted to nanoparticles was quantified by measuring absorbance in water (200 µL) at 260 nm and 560 nm with a microplate reader (Perkin Elmer). The nanoparticle concentration was estimated as described previously (Bonnet et al. 2018). To fit with this estimation, we approximated a molar extinction coefficient to 163200 M^{-1} cm^{-1} that considers

the epsilons of the different parts of the sequence corrected by their corresponding coupling yield.

Functionalized NPs were observed under transmission electron microscopy (TEM) using a Philips CM120 instrument operating at an accelerate voltage of 120 kV (Centre Technologique des microstructures, Lyon). Si-NPs were observed after deposition of 5 µL of diluted solution on a formvar-carbon coated copper grid and evaporation to dryness.

Results and discussion

3.1. Preparation and characterization of biotin-Si-NPs

Biotin-Si-NPs were prepared by an innovative solid-phase synthesis technology that allowed for a very high functionalization. Hydroxyl group was attached to the Si-NP surface by silanization to support the oligonucleotide linker synthesis on the NP surfaces, as previously reported (Bonnet et al. 2018; Farre et al. 2010). In the final step, biotin groups were grafted to the linker. TEM observations showed that NP morphology was stable during synthesis (Fig. 1A). Functionalized biotin-Si-NPs had an average size of 50 ± 3 nm, as estimated from TEM images (Fig. 1B). The absorbance measurements suggested that about 500 molecules of linker were attached to each NP, while dimethoxytrityl quantification indicated the biotin-coupling yield of 10 %, which quantified biotin to about 50 per NP (Fig. 1C).

A quite narrow size distribution of functionalized NPs was confirmed by DLS measurements. Si-NPs formed monodisperse aqueous solutions of particles with a hydrodynamic diameter (R_H) of about 90 nm. Conjugation of biotin with the linker moieties shifted R_H to about 120 nm (Fig. 1D). This increase is probably related to the hydration layer

around biotin units linked to arms bearing negatively charged groups. Biotin-Si-NP solutions were stable at 4°C for at least two months.

3.2. Concept of biotin-Si-NPs based DNA dot blot

The principle of the DNA dot blot method for *Campylobacter* detection enhanced with biotin-Si-NPs is described in Fig. 2. Initially, the target DNA was immobilized on a porous nylon membrane by the UV irradiation to enable crosslinking of DNA to the positively charged surface. Thereafter, the DNA probe CampyP3 labeled with biotin was allowed to hybridize with the target DNA. The hybridization was detected with a streptavidin-HRP conjugate in combination with a chemiluminogenic substrate luminol in the presence of the activator H_2O_2. In enhanced detection, the streptavidin-biotin sandwich enabled to attach biotin-Si-NPs to the DNA probe, and consequently to amplify the detection signal compared to a single biotin.

3.3. *Optimization of key parameters and analytical performances of the DNA dot blot sensor*

The time and temperature of hybridization of the DNA probe with its target were optimized as they can markedly influence the sensibility and selectivity of a DNA sensor. First, the hybridization of DNA was tested at room temperature, 44°C, 55°C and 65°C using complementary and non-complementary short ssDNA targets. Complementary DNA target sequence CP3 (positive control) was detected at all temperatures tested, but only at 65°C no non-specific binding was obtained with negative controls, a truncated target sequence (PR) and *E. coli* sequence (PE) (Fig. 3A). Second, hybridization was studied using 1 ng/µL CP3 at 65°C for different times from 1 h to overnight. The overnight incubation provided the highest level of hybridization and the maximal signal intensity.

Other parameters that may affect hybridization, such as buffer compositions or a paper support were also tested. The optimization criterion was the best signal-to-background ratio. The nylon membrane allowed for better immobilization of DNA compared to nitrocellulose. The effects of formamide (10, 25, 30, 35 and 50 %), DMSO (0 and 10%) and SDS (0, 1 and 10 %) in hybridization buffer were tested since these denaturing agents for double strained DNA may prevent nonspecific hybridization. The optimal hybridization buffer contained SDS, and no DMSO or formamide. We have found that addition of 1 % SDS enhanced the intensity of signals obtained and reduced the frequency of unspecific background staining. The effect of SSC concentrations (0.5x, 1x and 2x) in the washing buffer was also investigated. A 3-step washing procedure with two buffers (2xSSC, 0.1 % SDS and 0.5xSSC, 0.1 % SDS) leaded to elimination of the background signal while maintaining high light intensity of specific spots.

Detection of 1 ng/µL CP3 under the optimized conditions using various concentrations of streptavidin-HRP and biotin-Si-NPs allowed to select 25 ng/µL of streptavidin-HRP and 10^6 nanoparticles/mL for the chemiluminometric reaction. It is worth to note that chemiluminometric reaction itself does not significantly contribute to the background staining as the emission of light arises from the enzymatic reaction without any photonic excitation (Laios et al. 2010).

3.4 Calibration curve

The calibration curves were obtained from the quantification of chemiluminescent light intensity for different CP3 concentrations, taken from at least three independent dots per concentration. The linear range for the CP3 target was from 1 ng/µL to 0.1 ng/µL (with correlation R^2=0.98684), and from 6 pg/µL to 0.1 ng/µL (with correlation R^2=0.98935), for classical and biotin-Si-NP enhanced dot blot technique, respectively (Fig. 3B-D). The limit of

detection (LOD) of 0.08 ng/µL and 0.003 ng/µL for classical and enhanced dot blot, respectively, were calculated using 3 s/m formula, where 's' is a standard deviation of the blank solution and 'm' is a slope of the linear calibration graph. Taking into account the molecular weight of the CP3 of 14395.5, the calculated LOD was 5.5 nM and 0.2 nM, for classical and enhanced dot blot, respectively. Consequently, biotin-Si-NPs allowed for almost 30 fold increase in chemiluminescent signal intensity compared to the classical dot blot. The proposed biosensor showed comparable or enhanced performance than recently reported DNA sensors for *Campylobacter* detection where the detection limit was 0.5 ng/µL in (Fontanot et al. 2014), 0.37 ng/µL in (Manzano et al. 2015) and 0.09 nM in (Morant-Miñana and Elizalde 2015). The biotin-Si-NP enhanced dot blot reproducibility was estimated to 5 % according to the signal obtained for detection of the same concentration of CP3.

3.4. *SEM characterization of the biotin-Si-NPs dot blot*

SEM was applied to examine different steps of the detection in order to directly visualize biotin-Si-NPs on the surface of the paper carrying hybridized DNA. Nylon surface before DNA immobilization showed typical membrane structure, including the membrane pores (Fig. 4). After *C. jejuni* DNA cross-linking to the nylon, the surface was occupied by a large quantity of ssDNA. Compared to the surface of the bare membrane, the surface of membrane carrying DNA became irregular showing increased morphological heterogeneity. ssDNA molecules seemed to rest horizontally positioned over the surface (Fig. 4, right upper panel). The membrane with hybridized DNA carrying biotin-Si-NPs (final detection step) presented thicker fibril structures than that with only immobilized ssDNA molecules (Fig. 4, lower panel). Biotin-Si-NPs were easily detected on double strain DNA as small beads of about 50 nm diameter. Overall, SEM images confirmed the efficient DNA immobilization and

hybridization by revealing that nylon surface became more complex after each stage of the detection process and that hybridized DNA were decorated with nanoparticles of the expected size of 50 nm.

3.5. *Specificity and selectivity of detection*

To examine the selectivity and sensitivity of the biotin-Si-NPs dot blot, we tested various strains of *Campylobacter* spp. for inclusivity, and 17 other bacterial species and 1 yeast strain for exclusivity (Fig. 5). All strains were cultivated as monoculture and genomic DNA were extracted as explained in experimental part. Dot blot analyses were performed using 10 ng/µL of non-amplified extracted DNA. Before immobilization, all genomic DNA were denatured for 5 min at 95°C to allow double strain opening. CP3 at 0.1 ng/µL was used as an intern control to normalize signal intensities. The signal ratios for *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* over CP3 were higher than 1.0, with the membrane images exhibiting obvious dots. Moreover, the intensity obtained for *C. jejuni* and *C. coli* were about 4 times higher than that of the positive control. In contrast, the highest ratio among control DNA and CP3 was only 0.6 for *S. enterica* (background staining). Obtained results highlight the specificity of detection. In addition, the CampyP3 detection probe reacted with DNA of *C. jejuni* and *C. coli* more efficiently than with DNA of other *Campylobacter* spp. tested. Our results demonstrated than biotin-Si-NPs enhanced dot blot platform is sensitive enough to detect whole DNA extracted from most frequent *Campylobacter* spp. and could distinguish between *Campylobacter* and other bacteria. This specificity of the CampyP3 probe suggests that no nonspecific signal will be generated upon testing DNAs of competing total mesophilic flora in chicken meat.

3.6. *Campylobacter spp. detection in naturally contaminated chicken samples*

To demonstrate the capabilities of our developed biosensor for possible real-world applications, we have chosen to target the detection of *Campylobacter* in chicken meat samples. The results of plate count enumeration for background bacteria in five chicken samples are reported in Table 3. The ISO 10272-1:2006 indicated that two out of five samples were contaminated with *Campylobacter* (Table 4). Indeed, only isolates from selective media (mCCDA, Skirrow and Columbia agar) of samples C3 and C10 were confirmed for *Campylobacter* by the temperature growth and motility test.

In dot blot analysis, the CP3 template (0.1 ng/µL) was used as a positive control. The dot blot enhanced with biotin-Si-NP successfully detected two contaminated samples, C3 and C10 (Fig. 6). The test achieved a relative specificity of 100 % thanks to the high specificity of the CampyP3 detection probe. The proposed biotin-Si-NP enhanced dot blot method, thus, specifically detected low levels of naturally present *Campylobacter* after enrichment and could be considered for determination and detection of *Campylobacter* spp. in contaminated chicken meats. Compared to the official ISO 11272: 2006 method, the new DNA dot blot biosensor is less laborious, more cost-effective and time-saving.

Conclusion

This work describes the DNA dot blot method enhanced with biotin-Si-NPs for easy, fast and reliable detection of *Campylobacter* spp. in contaminated chicken meats. Dot blot test is widely used in molecular biology and genetics for detecting target DNA/RNA sequence. This paper-based hybridization can easily analyze multiple samples inexpensively in a high-throughput fashion but has limitation in a low sensitivity. We demonstrated that the association of highly functionalized biotin-Si-NPs with the chemiluminescent read-out of the dot blot enhanced signal about 30 times. Furthermore, biotin-Si-NPs are robust against

working temperature and stable in time. The LOD obtained was 3 pg/µL (0.2 nM). Such a low concentration of detected DNA are close to those that can be detected by qPCR (Manzano et al. 2018; Vidic et al. 2019). The hybridization of immobilized genomic DNA with the CampyP3 probe induced positive signals for different *Campylobacter* spp. responsible for human gastroenteritis, while no background staining was observed with control samples. The developed system is a promising tool for fast and cheap screening of poultry samples for the presence of *Campylobacter* since the detection is performed on bacterial DNA without a pre-amplification step. Furthermore, our dot blot can be applied on DNA extracted by different extraction methods and from various food matrices, as it is not sensitive to DNA polymerase inhibitors.

The proposed sensitive, miniaturized and multiplex paper-based test is simple to design and could be used by the food industry and regulatory agencies for the detection of other pathogens to monitor food quality. We believe that in the future it could be integrated into a Lab-on-the chip based biosensor that comprises an automatized DNA extraction protocol and a mobile phone camera (Kalligosfyri et al. 2019; Vidic et al. 2019).

CRediT authorship contribution statement

Priya Vizzini: Investigation, Visualization, Formal analysis, Writing - original draft. **Marisa Manzano**: Methodology, Re-sources, Supervision, Writing - review & editing. **Carole Farre**: Investigation, Formal analysis. **Thierry Meylheuc**: Investigation, Formal analysis. **Carole Chaix**: Methodology, Supervision, Formal analysis, Writing - review & editing. **Nalini Ramarao**: Supervision, Writing - review & editing. **Jasmina Vidic**: Conceptualization, Methodology; Supervision, Data curation, Resources, Validation; Project administration; Roles/Writing - original draft; Writing - review & editing.

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5. References

Batz, M., Hoffmann, S., Morris Jr, J.G., 2014. Disease-outcome trees, EQ-5D scores, and estimated annual losses of quality-adjusted life years (QALYs) for 14 foodborne pathogens in the United States. Foodborne pathogens and disease 11(5), 395-402.

Black, R.E., Levine, M.M., Clements, M.L., Hughes, T.P., Blaser, M.J., 1988. Experimental Campylobacter jejuni infection in humans. Journal of infectious diseases 157(3), 472-479.

Bonnet, R., Farre, C., Valera, L., Vossier, L., Léon, F., Dagland, T., Pouzet, A., Jaffrézic-Renault, N., Fareh, J., Fournier-Wirth, C., 2018. Highly labeled methylene blue-ds DNA silica nanoparticles for signal enhancement of immunoassays: application to the sensitive detection of bacteria in human platelet concentrates. Analyst 143(10), 2293-2303.

De Boer, P., Rahaoui, H., Leer, R., Montijn, R., Van der Vossen, J., 2015. Real-time PCR detection of Campylobacter spp.: a comparison to classic culturing and enrichment. Food microbiology 51, 96-100.

De Crozals, G., Farre, C., Hantier, G., Léonard, D., Marquette, C.A., Mandon, C.A., Marmuse, L., Louis, C., Toulmé, J.-J., Billotey, C., 2012. Oligonucleotide solid-phase synthesis on fluorescent nanoparticles grafted on controlled pore glass. RSC advances 2(31), 11858-11866.

Dincer, C., Bruch, R., Costa-Rama, E., Fernández-Abedul, M.T., Merkoçi, A., Manz, A., Urban, G.A., Güder, F., 2019. Disposable sensors in diagnostics, food, and environmental monitoring. Advanced Materials 31(30), 1806739.

ECDC, E.a., 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSa Journal 16(12), e05500.

Farre, C., Lansalot, M., Bazzi, R., Roux, S., Marquette, C.A., Catanante, G., Blum, L.c.J., Charvet, N., Louis, C., Chaix, C., 2010. Automated oligonucleotide solid-phase synthesis on nanosized silica particles using nano-on-micro assembled particle supports. Langmuir 26(7), 4941-4950.

Fontanot, M., Iacumin, L., Cecchini, F., Comi, G., Manzano, M., 2014. Rapid detection and differentiation of important Campylobacter spp. in poultry samples by dot blot and PCR. Food microbiology 43, 28-34.

Gosiewski, T., Ludwig-Galezowska, A., Huminska, K., Sroka-Oleksiak, A., Radkowski, P., Salamon, D., Wojciechowicz, J., Kus-Slowinska, M., Bulanda, M., Wolkow, P., 2017. Comprehensive detection and identification of bacterial DNA in the blood of patients with sepsis and healthy volunteers using next-generation sequencing method-the observation of DNAemia. European Journal of Clinical Microbiology & Infectious Diseases 36(2), 329-336.

Gosselin-Théberge, M., Taboada, E., Guy, R.A., 2016. Evaluation of real-time PCR assays and standard curve optimisation for enhanced accuracy in quantification of Campylobacter environmental water isolates. Journal of microbiological methods 129, 70-77.

Hermans, D., Pasmans, F., Messens, W., Martel, A., Van Immerseel, F., Rasschaert, G., Heyndrickx, M., Van Deun, K., Haesebrouck, F., 2012. Poultry as a host for the zoonotic pathogen Campylobacter jejuni. Vector-Borne and Zoonotic Diseases 12(2), 89-98.

Humphrey, T., O'Brien, S., Madsen, M., 2007. Campylobacters as zoonotic pathogens: a food production perspective. International journal of food microbiology 117(3), 237-257.

Jokerst, J.C., Adkins, J.A., Bisha, B., Mentele, M.M., Goodridge, L.D., Henry, C.S., 2012. Development of a paper-based analytical device for colorimetric detection of select foodborne pathogens. Analytical chemistry 84(6), 2900-2907.

Kalligosfyri, P.M., Sevastou, A., Kyriakou, I.K., Tragoulias, S.S., Kalogianni, D.P., Christopoulos, T.K., 2019. Smartphone-based chemiluminometric hybridization assays and quantitative competitive polymerase chain reaction. Analytica Chimica Acta 1088, 123-130.

Laios, E., Ioannou, P.C., Christopoulos, T.K., 2010. Gene Assays Based on Bio (Chemi) luminescence. Chemiluminescence and Bioluminescence, pp. 334-377. RSC Publishing, Cambridge, UK.

Liu, K.C., Jinneman, K.C., Neal-McKinney, J., Wu, W.-H., Rice, D.H., 2017. Simultaneous identification of Campylobacter jejuni, Campylobacter coli, and Campylobacter lari with smartcycler-based multiplex quantitative polymerase chain reaction. Foodborne Pathogens and Disease 14(7), 371-378.

Manzano, M., Cecchini, F., Fontanot, M., Iacumin, L., Comi, G., Melpignano, P., 2015. OLED-based DNA biochip for Campylobacter spp. detection in poultry meat samples. Biosensors and bioelectronics 66, 271-276.

Manzano, M., Viezzi, S., Mazerat, S., Marks, R.S., Vidic, J., 2018. Rapid and label-free electrochemical DNA biosensor for detecting hepatitis A virus. Biosensors and Bioelectronics 100, 89-95.

Masdor, N.A., Altintas, Z., Tothill, I.E., 2016. Sensitive detection of Campylobacter jejuni using nanoparticles enhanced QCM sensor. Biosensors and Bioelectronics 78, 328-336.

Morales-Narváez, E., Golmohammadi, H., Naghdi, T., Yousefi, H., Kostiv, U., Horak, D., Pourreza, N., Merkoçi, A., 2015. Nanopaper as an optical sensing platform. ACS nano 9(7), 7296-7305.

Morant-Miñana, M.C., Elizalde, J., 2015. Microscale electrodes integrated on COP for real sample Campylobacter spp. detection. Biosensors and Bioelectronics 70, 491-497.

Ricke, S.C., Feye, K.M., Chaney, W.E., Shi, Z., Pavlidis, H., Yang, Y., 2019. Developments in rapid detection methods for the detection of foodborne Campylobacter in the United States. Frontiers in Microbiology 9, 3280.

Russell, S.M., Doménech-Sánchez, A., de la Rica, R., 2017. Augmented reality for real-time detection and interpretation of colorimetric signals generated by paper-based biosensors. Acs Sensors 2(6), 848-853.

Scallan Walter, E.J., Crim, S.M., Bruce, B.B., Griffin, P.M., 2020. Incidence of Campylobacter-associated Guillain-Barre Syndrome estimated from health insurance data. Foodborne Pathogens and Disease 17(1), 23-28.

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors–occurrence, properties and removal. Journal of applied microbiology 113(5), 1014-1026.

Trinh, K.T.L., Stabler, R.A., Lee, N.Y., 2020. Fabrication of a foldable all-in-one point-of-care molecular diagnostic microdevice for the facile identification of multiple pathogens. Sensors and Actuators B: Chemical, 128057.

Vidic, J., Manzano, M., Chang, C.-M., Jaffrezic-Renault, N., 2017. Advanced biosensors for detection of pathogens related to livestock and poultry. Veterinary research 48(1), 11.

Vidic, J., Vizzini, P., Manzano, M., Kavanaugh, D., Ramarao, N., Zivkovic, M., Radonic, V., Knezevic, N., Giouroudi, I., Gadjanski, I., 2019. Point-of-need DNA testing for detection of foodborne pathogenic bacteria. Sensors 19(5), 1100.

Vizzini, P., Braidot, M., Vidic, J., Manzano, M., 2019. Electrochemical and Optical Biosensors for the Detection of Campylobacter and Listeria: An Update Look. Micromachines 10(8), 500.

WHO, 2020. World Health Organization. Campylobacter. Available online: https://www.who.int/news-room/fact-sheets/detail/campylobacter.

Yang, X., Kirsch, J., Simonian, A., 2013. Campylobacter spp. detection in the 21st century: A review of the recent achievements in biosensor development. Journal of microbiological methods 95(1), 48-56.

Table 1.

List of oligonucleotides used in this study.

DNA sequences	
Detection probe (CampyP3 probe)	5'-[biotin] TAG TGG CGC ACG GGT GAG TAA GGT ATA GTT AAT CTG-3'
Complementary probe (CP3 target)	5'-CAG ATT AAC TAT ACC TTA CTC ACC CGT GCG CCA CTA-3'
Non-complementary probe PR (rundom)	5'- CGT GCG CCA CTA CAG ATT ACC TTA AAC TAT CTC ACC-3'
Non-complementary probe PE (*E. coli* target PE)	5'- GAA AAG CGC GCT GGT GAA AAA AGA GAT GCG CGT CTT-3'

Table 2.

Campylobacter spp. and control bacterial strains used in this study.

Microorganism	Collection code
C. jejuni	DSM[a] 4688
C. coli	DSM[a] 24155
C. lari	DSM[a] 11375
C. upsaliensis	DSM[a] 5365
H. pylori	DSM[a] 7492
H. pylori	ICS[b]
Arc. butzleri	DSM[a] 8739
C. cryaerophila	DSM[a] 7289
L. monocytogenes	ATCC[c] 7644
L. innocua	DSM[a] 20649
L. seelligeri	DSM[a] 20751
L. marthii	DSM[a] 23813
L. welshimeri	DSM[a] 15452
L. ivanovii	DSM[a] 52491
S. aureus	DIAL[d]
B. cereus	DSM[a] 4282
B. cereus	DIAL[c] RC3
S. enterica	DSM[a] 9378
E. coli	DISTAM[e]
Lb. plantarum	ATCC RAA[b] 793
B. subtilis	DSM[a] 4181
S. cerevisiae	ATCC[b] 36024

[a]DSM: Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (Braunschweigh, Germany); [b]ATCC: American Type Culture Collection (Manassas, VA, USA); [b]DIAL : Dipartimento di Scienze e Tecnologie Alimentari (Udine, Italy); [d]DISTAM: Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche (Milan, Italy); [e]Isolated from Clinical samples (Hospital of Udine, Italy).

Table 3.

Plate count data of chicken (C) samples expressed as log of Colony Formin Unit (CFU/g).

Sample	Total viable count	Enterobacteriaceae	Coliforms	*E. coli*	Yeasts	Molds
C3	5.3	3	1.1	2.9	2.9	0.7[1]
C4	6.7	4.1	3.5	3.7	5	0.7[1]
C5	7.2	4.3	3.8	3.1	5.8	0.7[1]
C6	5.8	3.2	3.4	2.9	4.8	0.7[1]
C10	4.1	3.1	2.9	1.9	2.2	0.7[1]

[1], under the limit of detection

Table 4.

The presence /absence of *Campylobacter* spp. in chicken (C) samples assessed with the ISO 10272-1:2006 protocol.

Sample	mCCDA	SKR	CBA	Confirmation Absence/presence		Oxidase	Motility	Results
				41.5°C	25°C			
C3	+	-	+	-	-	+	+	+
C4	+	-	+	+	+	+	-	-
C5	+	-	+	+	+	+	-	-
C6	+	-	+	+	+	+	-	-
C10	+	-	+	-	-	+	+	+

mCCDA, modified charcoal and deoxycholate agar selective medium; SKR, Skirrow selective medium; CBA, Colombia blood agar.

Figures:

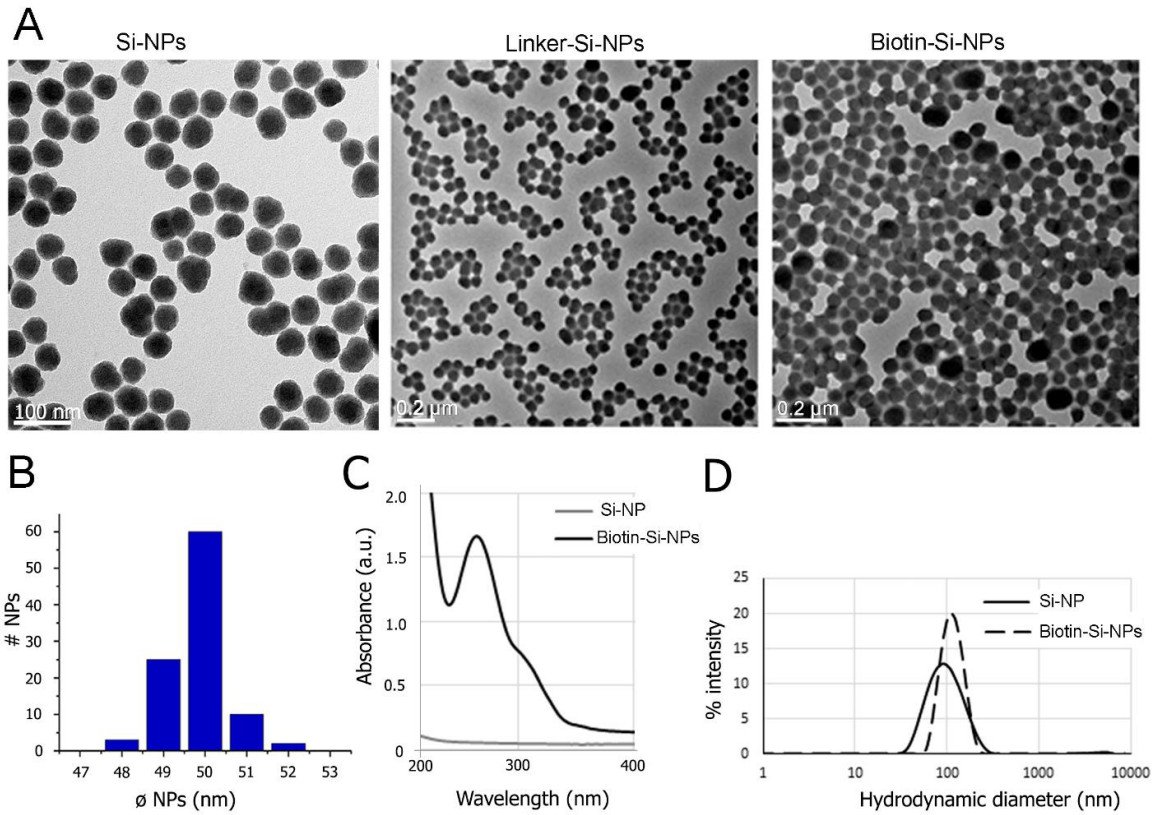

Figure 1. (A) TEM images of Si-NPs before and after functionalization with linker and biotin. (B) Histogram showing the average particle size 50 ± 3 nm before functionalization. (C) Absorbance spectra of Si-NPs (before functionalization) and biotin-Si-NPs (after functionalization). (D) DLS size plots of native and biotin functionalized Si-NPs in water.

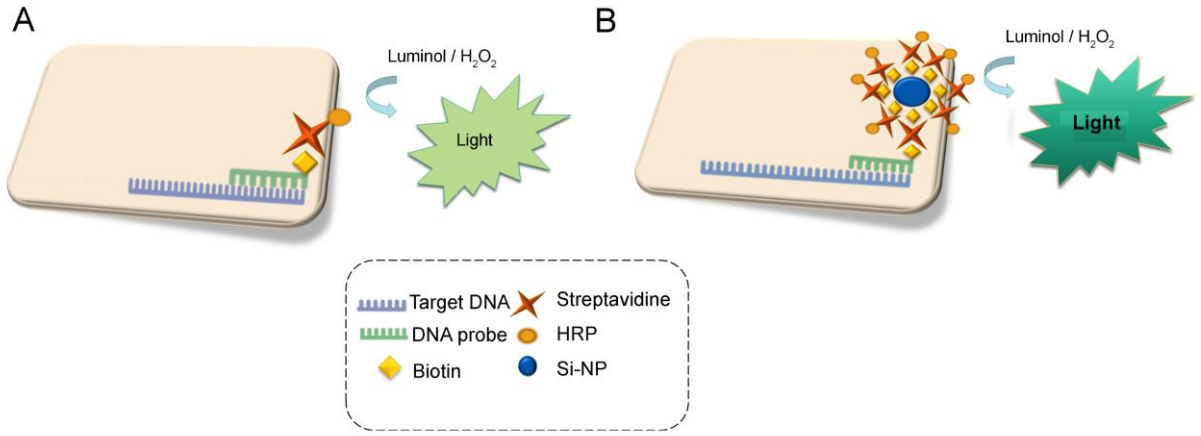

Figure 2. Schematic representation of *Campylobacter* detection based on paper-based DNA hybridization with a complementary biotinylated probe, and a streptavidin-HRP read-out through dot blot (A). The signal was amplified by using highly functionalized biotin-Si-NPs instead of a single biotin (B).

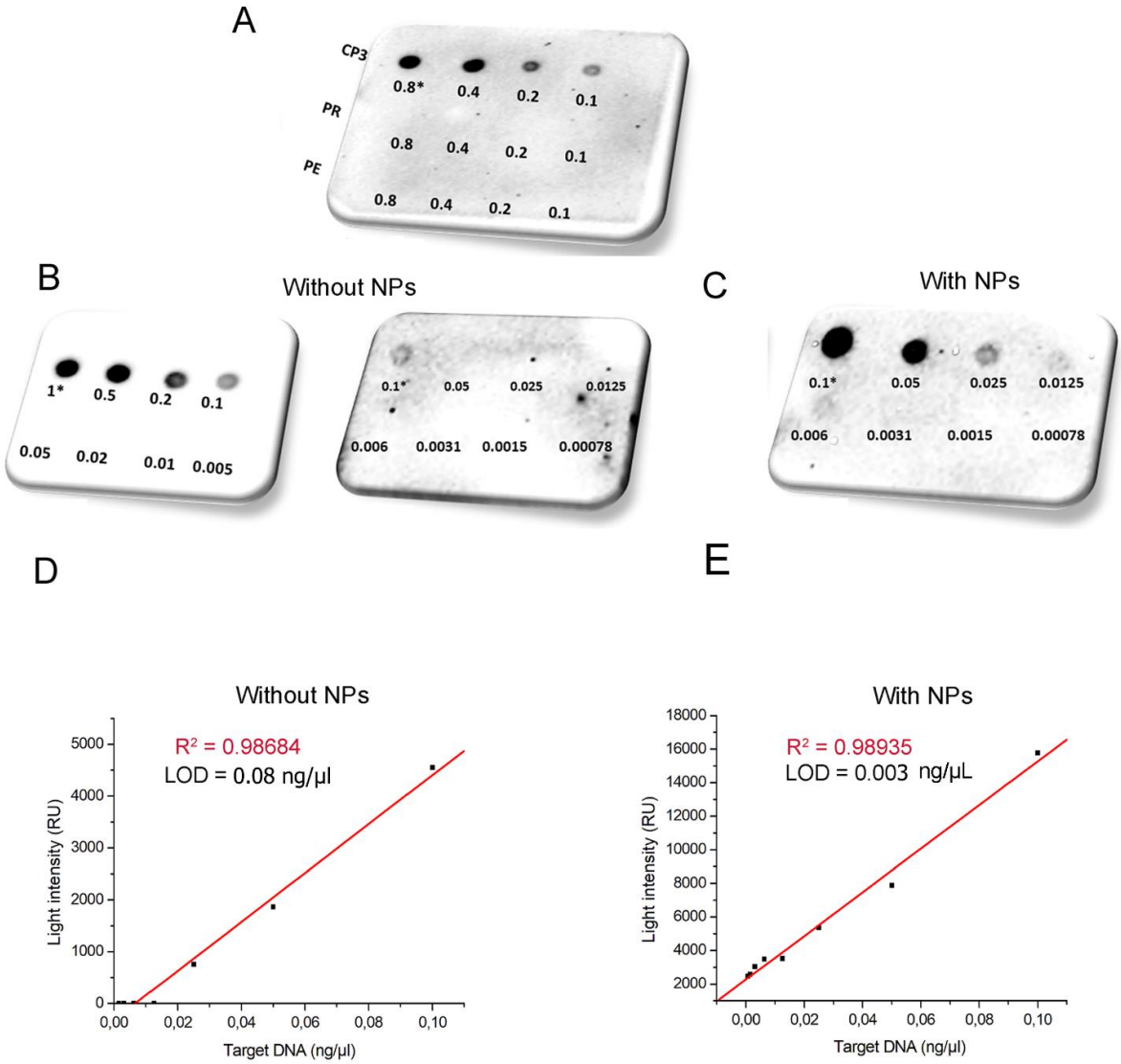

Figure 3. (A) Dot blot detection of *Campylobacter* DNA sequence with biotin-Si-NP enhanced read-out. Note that no signal was obtained with a truncated *Campylobacter* sequence (PR) nor with an *E. coli* control sequence (PE). (B) Conventional dot blot detection of biotin labeled CampyP3 probe using dilution of complementary *Campylobacter* sequence CP3 ranging from 1ng/ µL to 78 fg/µL. (C) Enhanced dot blot detection of CP3 (0.1 ng/µL – 78 fg/µL) using CampyP3 probe. (D, E) Corresponding calibration curves were obtained by plotting the chemiluminescent signal intensity of dots in a function of CP3 template concentrations.

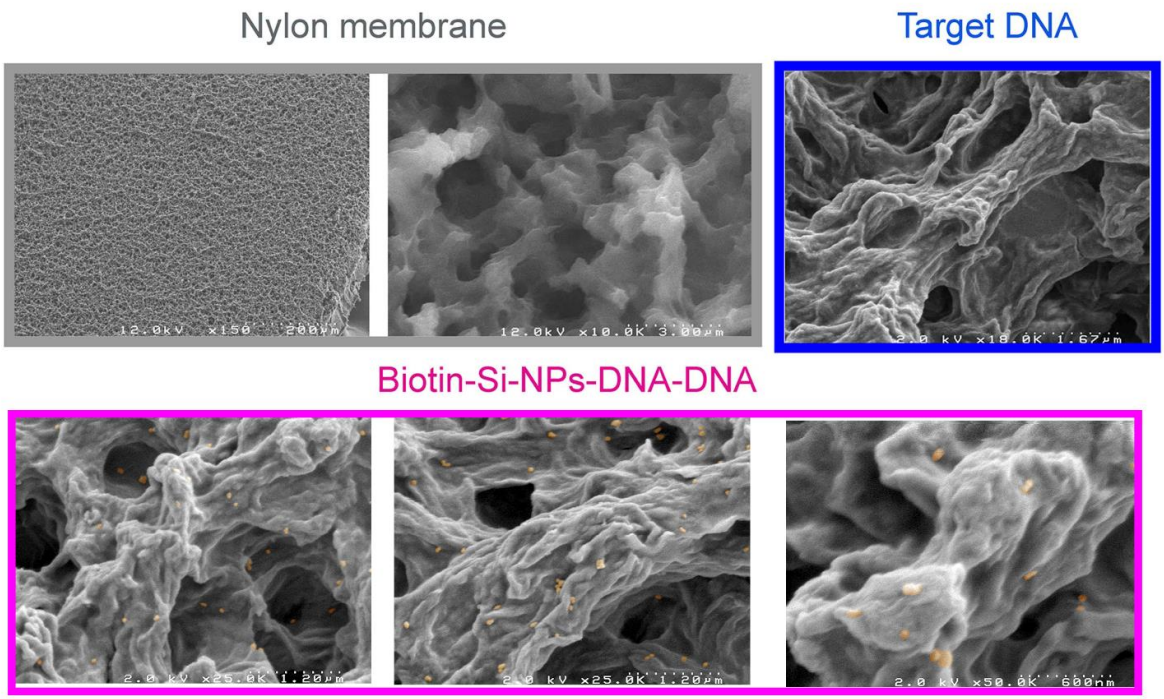

Figure 4. SEM images of basic nylon membrane, nylon membrane functionalized with a *C. jejuni* DNA, and nylon membrane with CampyP3 hybridized with *C. jejuni* DNA and revealed with biotin-Si-NPs. Note that the size of visualized circular beads of about 50 nm (yellow shading) correspond to the estimated NPs size in Fig 1B.

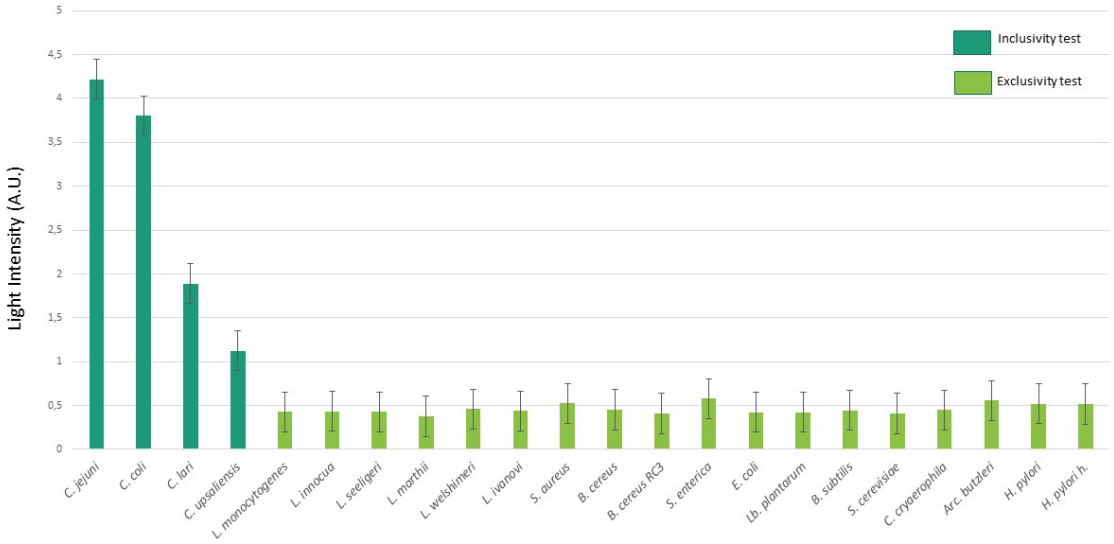

Figure 5. Inclusivity and exclusivity test results of the enhanced dot blot biosensor in pure bacterial cultures. Signal intensity were normalized using an intern control (CP3 template). Error bars represent the standard deviation of the mean from triplicates.

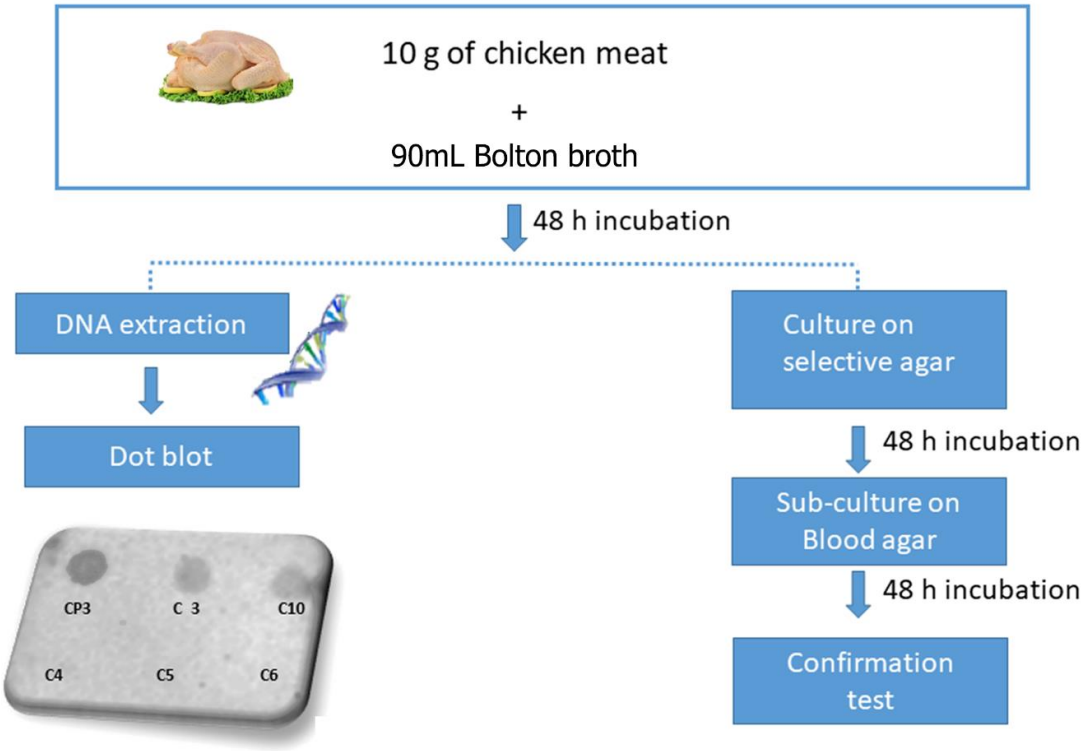

Figure 6. Schematic diagram of *Campylobacter* detection from chicken samples using the enhanced dot blot and the official ISO 10272-1:2006 method. The ISO method involved steps performed to obtain results in Table 4. Dot blot membrane shows representative dot blot results obtained with naturally infected chicken samples (C3 and C10) and non-infected chicken samples (C4, C5 and C6). CP3 template sequence (0.1 ng/µL) was used as a positive control.