ACTA SCIENTIFIC MICROBIOLOGY (ISSN: 2581-3226)

Volume 1 Issue 10 October 2018

Research Article

Nucleic-Acid Lateral Flow Assay Optimization with Different Gold Nanoparticle Size for Detection of Pathogen after PCR, Using *L. Monocytogenes* as Model

Pérez-Etcheverry Diana[1], Pérez-Neira Sandra[1], Brugnini-Osimani Giannina[2], Rufo-D`Addario Caterina[2] and Lorenzo-Ferreiro Carmen[1]*

[1]*Area de Biotecnologia del Instituto Polo Tecnologico de Pando - Facultad de Quimica, Universidad de la Republica, Canelones, Uruguay*

[2]*Area de Alimentos y Nutricion Polo Tecnologico de Pando - Facultad de Quimica, Universidad de la Republica, Canelones, Uruguay*

***Corresponding Author:** Lorenzo-Ferreiro Carmen, Area de Biotecnologia del Instituto Polo Tecnologico de Pando - Facultad de Quimica, Universidad de la Republica, Canelones, Uruguay.

Received: August 24, 2018; **Published:** September 20, 2018

Abstract

The aim of this work was to create a fast, sensitive and robust test, based on lateral flow assay using gold nanoparticles conjugated with DNA probe to be used as tool for microbial detection in relation to food safety or clinical diagnosis. This platform was designed to contribute reducing the time of diagnosis based on culture assay of pathogens and facilitate the use in point of care. The capture gold nanoparticles on the test and control line of the lateral flow strip cause the characteristic precipitation bands visible to the naked eye, easy to manipulate and of easy interpretation. Here, we report the optimization of three parameters associated to the sensitivity and reproducibility of the strips. Gold nanoparticles size, concentration of reporter probe and lateral flow membrane characteristics were evaluated and the combination that performed with better results was used to construct and test lateral flow strips. The platform was designed using *Listeria monocytogenes* as the model microorganism, due to its pathogenicity, its ubiquity and its concern in the food industry. *Listeria monocytogenes* is one of the most virulent food borne pathogens, it can be found in fresh or processed foods, either from animal or vegetal origin. It causes listeriosis, a rare but severe condition, which may be fatal in high-risk individual. The standard method for detection of *L. monocytogenes* and *Listeria* spp. following ISO 11290-1:2017 is complex and time-consuming [1]. It requires 24 and 48 hrs of enrichment, followed by a variety of biochemical tests for identification. To detect the presence of pathogens it is necessary a first step of selective enrichment, followed by an identification step to avoid false negative results. Here, we report the construction of a rapid, disposable nucleic acid based biosensor for specific detection of *L. monocytogenes* after a PCR amplification step that responds in 10 minutes. Our DNA dipstick was evaluated using a *L. monocytogenes* fragment obtained after PCR amplification. No need of pre-hybridization or purification steps is necessary; PCR product can be directly applied at the sample pad, only requiring 1 min of denaturation step at 95ºC.

Our approach shows to be a useful tool for detection of *L. monocytogenes* or other pathogens, after necessary step of amplification.

Keywords: Oligochromatography; Diagnostic; DNA Probes; Lateral Flow Assay; Gold Nanoparticles*; L. monocytogenes*

Abbreviations

LFA: Lateral Flow Assay; POC: Point of Care; PCR: Polymerase Chain Reaction; SSC: Saline Sodium Citrate Buffer; DNA: Deoxyribonucleic Acid

Introduction

Along the history of development of methods for diagnosis, a challenge of great concern was to provide trustworthy point of care (POC) devices for clinical diagnosis or control point at industrial facilities, aiming to minimize decision taking time. Ideal POC

tests are characterized by easy operational procedures, ability to generate rapid results and relatively low cost. Lateral Flow Assay (LFA) has become the most used platform for POC; it is a one-step analysis, provides prompt results in very short time [2], requires low sample volume, no need of expert personnel and low cost of operation [2-6]. Colloidal gold conjugation with biomolecules has provided an excellent platform for detection of a variety of target analytes [6,7]. Additionally, different formats can be adopted in LFA, competitive, non- competitive assay, antibody based assay, oligonucleotide based assay among others [8]. Since 1980´s [9], a variety of immune-chromatographic test were successfully developed and commercialized. Conventional LFA is performed over a single strip, which includes a sample pad membrane, a conjugate pad membrane, a nitrocellulose membrane and an absorbent pad, all of them assembled on a unique plastic backing [7]. The strip contains all reagents immobilized at different parts; once the liquid sample is applied at the sample pad; the flow allows activating all reagents and positive reaction become visible at naked eye by deposition of gold labeled conjugate. Dipstick-type DNA biosensor is a promising tool for use in a broad range of applications where the detection of typifying amplicons is required [10]. In this case, DNA probes are immobilized in the nitrocellulose membrane in the test and control lines. The method is equipment independent and faster than agarose gel electrophoresis analysis of PCR product. For this reason resulted of great value for clinical and non-clinical utilization, notably in small labs with minimal equipment [10-14]. An interesting field of application is food and water quality assurance and safety monitoring [15-18]. Food safety requires not only good manufacturing procedures but also reliable methods to detect the presence of undesirable microorganism during the industrial process. Conventional methods as culture-based methods are usually the gold-standard methods, although they perform with good sensitivity and do not require sophisticated equipment, they are time consuming, and stringent conditions are requisite to maintain specimen viability, as well as skilled personnel is required [19]. Therefore, PCR followed by a simple step of LFA based on oligonucleotide identification is gaining popularity as simple, robust, and fast detection of microorganism of interest.

In this work we used a fragment of the pathogen *L. monocytogenes* as a model to present a disposable LFA-DNA biosensor based on oligonucleotide functionalized gold nanoparticles for fast, sensitive and POC detection of PCR product. *L. monocytogenes* is the second pathogen in importance in causes of death transmitted by food [20-25]. Sandwich-type DNA hybridization reaction is performed directly after applying the sample on a lateral flow strips without pre-hybridization step. Our LFA resolving time is only 10 min, following PCR, and then qualitative perception can be performed by observing the color change in the test line. The sensitivity of our biosensor evaluated against synthetic single-stranded DNA probe reached 25 fmol in 100 µL of sample. The utility of the biosensor was evaluated by assaying real samples subjected to PCR for amplification of typifying fragment and subsequently applied to the dipstick.

Materials and Methods

Reagents and apparatus

Bovine serum albumin (BSA), $HAuCl_4$, sucrose, triethylamine, Tween 20, Triton-X, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), sodium citrate, streptavidin from *Streptomyces avidinii*, and all other common chemicals were obtained from Sigma-Aldrich. Saline Sodium Citrate (SSC) was used as hybridization buffer. Solutions were prepared with ultrapure (> 18 MΩ) water from a Millipore Milli-Q water purification system (Millipore, USA). Laminated membrane cards of fast and medium speed were used: Hi-Flow™ Plus 75 (Millipore, USA) and UniStart CN 140 (Sartorius, Germany), glass fibers and cellulose fiber sample pads were purchased from Millipore.

DNA probes and synthetic target were synthesized by Macrogen Inc. Three single-stranded oligonucleotides were designed to detect the target, schematically presented in figure 1. An Isoflow™ Dispenser (Imagene Technology Inc, USA) and the Guillotine Model Index-Cut I from APoint Technologies, Inc USA, were used in this study.

Preparation of DNA conjugates with gold nanoparticles (AuNP) of different size

Conjugates of different AuNP size carrying different DNA concentration were prepared essentially as described by Mao X., *et al.* [26] with minor adaptations. Firstly, gold nanoparticles (AuNP) with average diameter 2 nm, 12 nm or 30 nm were prepared essentially as described by Hermanson GT [27]. Solutions of 4% $HAuCl_4$ or 1% $HAuCl_4$ were prepared in ultrapure water. In brief, to prepare the 2 nm AuNP, 375 µL of the 4% chloroauric acid solution plus 500 µL of 0.2 M K_2CO_3 were added to 100 mL of ultrapure water, cooled and maintained on ice. Then, 5 mL of freshly prepared $NaBH_4$ (0.5 mg/mL) were added in five aliquots of 1 mL to the chloroauric acid/carbonate suspension with vigorous stirring and further stir on ice for 5 minutes. The resulting solution

was reddish-orange. To prepare the 12 nm gold particle solution, 4 mL of 1% $HAuCl_4$ and 4 mL of 0.1M K_2CO_3 were added to 100 mL of ultrapure water as described above. After that, 1 mL of 7% sodium ascorbate solution in ultrapure water was added with vigorous stirring on ice, avoiding the temperature to rise. The volume was adjusted to 400 mL with ultrapure water and the mixture was brought to boil and reflux until the color of the suspension turned to red. Finally, the suspension was allowed to cool at room temperature and concentrated by centrifugation as 10000 rpm for 15 minutes (Sigma 3-30K, USA). The 30 nm gold particle solution was prepared at follows: 3 mL of a 1% sodium citrate solution, in ultrapure water, were added to a boiling solution of 0.5 mL of 4% $HAuCl_4$ in 200 mL of ultrapure water, with rapid mixing. Boiling and reflux was sustained for 30 min and then the suspension was cooled to room temperature. The color of the resulting suspension was red. The suspension was concentrated by centrifugation at 10000 rpm for 15 minutes (Sigma 3-30K, USA). The prepared colloids of AuNP were characterized by absorption at 520 nm (UV/visible Spectrophotometer Ultrospec 3100pro, Amersham Biosciences) and by particle size (Malvern Zetasizer Nano ZS, Malvern Instruments Limited UK). Concentrated AuNP suspensions were used to prepare DNA-AuNP conjugates.

Nine conjugates of DNA-AuNP were developed by combining the three different AuNP sizes with three DNA amounts. For that purpose, activation of the thiolated DNAprobe_3 was firstly achieved as described by Mao., *et al*. [26]. Briefly, 0.1 OD/ 1 OD / 2 OD of DNAprobe_3 was mixed with 0.2 µL/ 2 µL / 20 µL, of triethylamine and 0.77 mg/ 7.7 mg / 77 mg of DTT and incubated for 30 minutes at room temperature. The excess DTT was removed by washing four times with ethylacetate solution. After that, 20 µL of the activated DNAprobe_3 were added to 2 mL of the concentrated AuNP solution of corresponding size and incubated at 4ºC for 24 hrs. Later, NaCl and SDS were added to final concentration of 75 mM and 0.01% respectively and maintained at 4ºC for another 24 hrs. The third day the samples were centrifuged at 5000 rpm for 5 minutes (Sigma 3-30K, USA), the supernatant having the excess of reagents was discarded and the pellet dispersed in 1 mL of a buffer containing 20 mM Na_3PO_4, 5% BSA, 0.25% Tween 20 and 10% sucrose. The same procedure was followed using all three AuNP solutions and activated DNA samples containing 0.1 OD, 1 OD or 2 OD.

Construction and trial of the oligobiosensor

DNAprobe_1 and DNAprobe_2 were conjugated to streptavidin in a molar ratio of 4:1 (biotin: streptavidin), mixing 30 µL of 200 mM corresponding biotinylated probe with 103 µL of 1 mg/ mL streptavidin solution. The mixtures were incubated for 30 min at room temperature and the volume was adjusted to 500 µL with ultrapure water. Streptavidin bound biotinylated capture probes DNAprobe_1 (test line) or DNAprobe_2 (control line) were applied on the nitrocellulose membrane using an Isoflow™ Dispenser, dispense speed 100 mm/sec, dispense rate 0.2 µL/mm. The membrane was then dried at 37 ºC for 1h and stored at room temperature until final use. The sample pad was saturated with 0.05 M Tris-HCl, 0.25% Triton X-100, 0.150 M NaCl (pH 8.0) and then dried at 37ºC. The conjugates were dispensed onto the fiber glass conjugate pad in one pass, till saturation, using the atomizer nozzle of the Isoflow™ Dispenser and dried at 37ºC for 1h. All the pads were assembled on a plastic adhesive backing, each one overlapping 3 mm to ensure the solution migration during the assay. Strips were cut at 3 mm with Index-Cut I. The so constructed strips were evaluated by applying at the sample pad, either synthetic single-stranded DNA oligonucleotide or PCR product, diluted in 4X SSC buffer in a final volume of 100 µL, and waited up to 10 minutes to read the results. PCR reaction was set-up as described by Barbau., *et al*. [28] for the amplification of the DNA segment used in this work, which is specific for *L. monocytogenes* typification. *L. monocytogenes* ATCC 19111 or *L. innocua* ATCC 33090 were used as templates; negative control was performed with ultrapure sterile water.

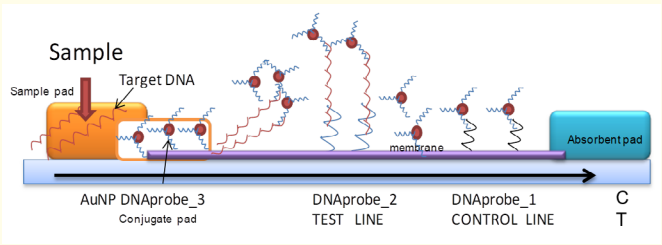
Results and Discussion

Principle of Measurement

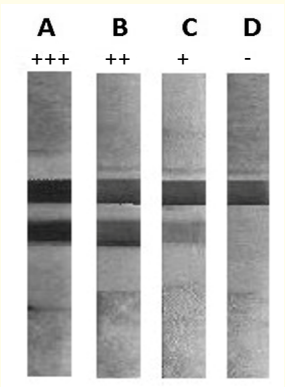
A schematic diagram illustrating the procedure of the oligonucleotide detection is shown in figure 1. The reporter DNAprobe_3 conjugated to AuNP is complementary to the 3´ ends of the target oligonucleotide in the sample; thus, the reporter conjugated with gold nanoparticles, hybridizes with the target DNA present in the sample solution that passes the conjugate pad and rehydrates it. Both the hybrid and free conjugate continue to migrate towards the Test zone, where the DNAprobe _2 is immobilized. DNAprobe_2 is complementary to the 5´ ends in the target oligonucleotide and forms a sandwich with the hybrid conjugate in the test line, generating a line visible to the naked eye. The excess of conjugate continues to migrate, then is captured by hybridization with the immobilized DNAprobe_1 in the control line, which is complementary to the 3´ ends of the reporter probe conjugated with AuNP, developing the second characteristic precipitation line in the Control zone. In the absence of target DNA in the sample, no accumulation of AuNP in the test line would take place, giving as a result a unique band in the control line, the appearance of this line assures that the strip worked properly. Such biosensor can be used in qualitative

analysis, by the simple fact of observing the development of a line in the test line as illustrated in figure 1.

(A)



(B)



(C)

Target DNA 5´- 3´:

ATCGAAAAGAAACACGCGGATGAAATCGATAAGTATATA-CAAGGATTGGATTACAATAAAAACAATGTA

DNAprobe_1: 5´-Biotin- ACAAGGATTGGATTACAATAAAAACAAT-GTA- 3´

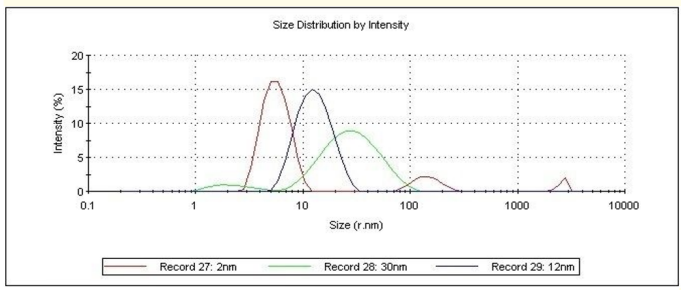
DNAprobe_2: 5´-TTATCGATTTCATCCGCGTGTTTCTTTT- Biotin- 3´

DNAprobe_3: 5´-Thiol C6S-S -TACATTGTTTTTATTGTAATCCAATCC-TTGT-3´

Figure 1: (A) Illustration of the principle of measurement for oligochromatography when target DNA is in the sample. (B) Photo image of four strips with different line intensity to the naked eye in the test line, defining line intensity as: +++ in line A ++ in line B + in line C; line D shows control strip without target DNA in the sample. (C) DNA oligonucleotide sequences of the target DNA and probes. Identity is denoted underlined, complementary regions are written in same colour blue/orange.

Optimization of assay parameters

In order to improve assay performance, three parameters were evaluated to find the best combination for the creation of strips. We investigated the effect of the size of gold nanoparticles on the response, in combination with the amount of the reporter DNA probe immobilized on them, maintaining the conjugate at saturation in the strips. The conjugates size alters the migration speed of the conjugate on the nitrocellulose membrane, affecting the lapse of time for hybridization. We prepared and compared the performance of three variants of AuNP with diameters of 2, 12 and 30 nm. Characterization of monodispersity and size distribution of the particles is shown in figure 2. Preparations of 12 and 30 nm size have good monodispersity and are of expected size. The suspension of 2 nm shows low degree of polydispersity but having a main peak (86.2% intensity) of approximately 5 nm size.



		Size (r.nm):	% Intensity	Width (r.nm):
2 nm	Peak 1:	5,756	86,2	1,620
	Peak 2:	146,0	10,9	39,39
	Peak 3:	2640	2,9	209,6
		Size (r.nm):	% Intensity	Width (r.nm):
12 nm	Peak 1:	13,37	100,0	4,821
	Peak 2:	0,000	0,0	0,000
	Peak 3:	0,000	0,0	0,000
		Size (r.nm):	% Intensity	Width (r.nm):
30 nm	Peak 1:	32,15	92,9	18,70
	Peak 2:	2,264	7,1	0,9360
	Peak 3:	0,000	0,0	0,000

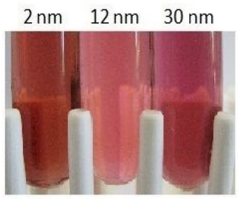


Figure 2: (A) Graphical analysis of particle size distribution obtained at the Malvern Zetasizer Nano ZS (Malvern Instruments Limited UK) for the three preparations of gold nanoparticles: red line, 2 nm particles, purple line 12 nm particles, green line 30 nm particles. (B) Data table of peaks, indicating size (nm), intensity of signal (%) and with of peak. (C) Photo image showing the different colors for three colloidal suspensions obtained.

Another consideration to have in mind to overcome the short hybridization time of this type of assay, is the amount of DNA probes attached to the gold nanoparticle. Theoretically, the more quantity of probes in the conjugate, the more efficient would be the hybridization. To study this effect, conjugates carrying 0.1 OD, 1.0 OD or 2.0 OD of DNAprobe_3 were prepared. All AuNP

DNAprobe_3 conjugates were assayed versus a synthetic DNA target in lateral flow strips prepared with DNAprobe_2 immobilized in the test line. The third criterion in consideration was the membrane. It is well known that physical and chemical attributes of the membrane affect the capillary flow properties, influencing reagent deposition, assay sensitivity and assay specificity among other parameters. The capillary flow rate is the speed at which a sample front moves along a membrane strip when liquid is introduced at one end. Typically expressed as a multiple of sec/cm, the capillary flow is governed by the membrane pore size, pore size distribution and porosity. We evaluated two nitrocellulose membranes, a fast flowing membrane Hi-Flow™ Plus 75, with a flow rate of 56 - 94 sec/4 cm, suitable for a quick response test, and a medium time-to-signal membrane UniStart CN 140 with a flow rate of 90 - 150 sec/4 cm. All the AuNP conjugates were evaluated in both membranes, using 0, 1, 10, 100 and 10000 nM target DNA in the sample. Sample was applied in a 4X SSC buffer. After ten min, the intensity of the positive responses was noted as "+", "++" or "+++" according to the intensity of the band at the test line zone, as illustrated in figure 1. Results are summarized in table 1. It can be seen that the response is highly dependent of the amount of immobilized probe. The response increases in intensity and sensitivity when DNA concentration in the conjugate increased from 0.1 to 1.0 OD for both membranes but decreases with excess of probes.

	HIFLOW PLUS 75				UniStart CN 140		
	reporter DNAprobe_3 (OD)				reporter DNAprobe_3 (OD)		
DNA in the sample (nM)	0,1	1	2	AuNp (nm)	0,1	1	2
10000	-	-	-	2	-	-	-
100	+	+++	-		+	+++	++
10	-	+++	-		-	+++	-
1	-	+++	-		-	++	-
0	-	-	-		-	-	-
10000	-	-	-	12	-	-	-
100	+++	+	-		+++	+++	++
10	+	+	-		++	++	-
1	-	+	-		-	++	-
0	-	-	-		-	-	-
10000	-	-	-	30	-	-	-
100	+++	+++	-		+++	+++	++
10	+	++	-		++	+++	+
1	-	-	-		+	++	+
0	-	-	-		-	-	-

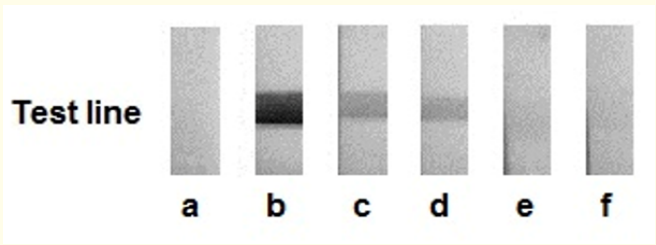
Table 1: Summary of the performance of the different dipsticks constructed with HIFLOW PLUS 75 membrane or UniStart CN 140 membrane and the nine conjugates developed with AuNP of 2, 12 or 30 nm carrying reporter probe in quantities of 0.1, 1 or 2 OD. 100 µL of sample containing 0, 1, 10, 100, 10000 nM synthetic target DNA were applied to each strip. Intensity of the bands was recorded as defined and illustrated in figure 1.

The time of hybridization depends on the migration speed, which is influenced by the size of AuNP and the constitution of the membrane. Particles of larger diameter migrated at slower speed resulting in longer assay time (10 minutes), but we found that in combination with strips having the slower flow membrane, favor the hybridization interval, and befall higher response signals. The strips constructed with the membrane of fastest flow, though the bands were developed faster, resulted in weaker response signals. When 1 OD of reporter was immobilized onto any of the AuNP, the target was revealed in a range of 1 to 100 nM with scarcely any differences in intensity when using the UniStart CN 140. Amount of 10000 nM is out of range of this biosensor, bands are not visible as free molecules migrate faster than hybridized conjugate and, being in excess, saturate the capacity of capture of the probe immobilized at the test line.

Evaluation of biosensor performance

We assayed the sensitivity of the best option for construction of the biosensor: 12 nm gold nanoparticles carrying 1 OD of reporter probe (at saturation) and UniStart CN 140 membrane. Samples containing 1 nM to 0.06 nM synthetic DNA target in 100 ul of 4X SSC buffer were applied and detected, with the test line being visible to the naked eye at concentrations as low as 0.25 nM. The detection limit corresponds to 25 fmol in the 100 µL sample solution in 10 minutes assay time (Figure 3). Photo image of the strips was analyzed with Doc ItLS software to calculate the intensity of the bands, and these values were plotted using GraphPad Prism™ software. Performance of the biosensor in realistic application was also evaluated. For this purpose, PCR reaction was set-up as described by Barbau., *et al.* [28] for the amplification of a well-defined segment, which is specific for *L. monocytogenes* typification. The resulting amplification mix was heated at 95ºC for 1 minute and subsequently applied onto the sample pad in 4X SSC buffer, 100 µL final volume. We found that strips worked satisfactory after a simple step of denaturation at 95ºC, without any additional treatment. In figure 3 it is shown an example where it can be appreciated that a line of precipitation is formed in the test line when the PCR product mix was applied onto the sample pad (Figure 3C, b), in the same manner to the positive control strip, which was generated using the single stranded synthetic target DNA added to the sample pad (Figure 3C, a). Specificity of the assay was confirmed by a negative response when control PCR product mix without template was tried, showing no interference due to components of the mix (Figure 3C, d). Absence of cross-reactivity with *L. innocua* was verified (Figure 3C, c). All PCR were run in triplicates.

(A)



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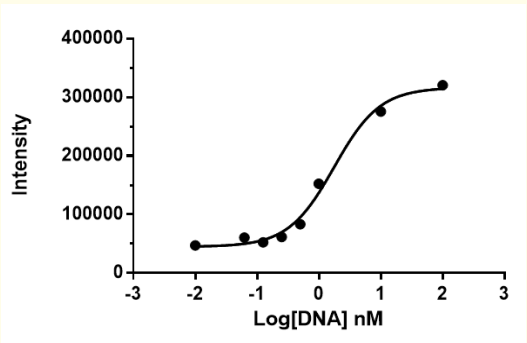

(C)

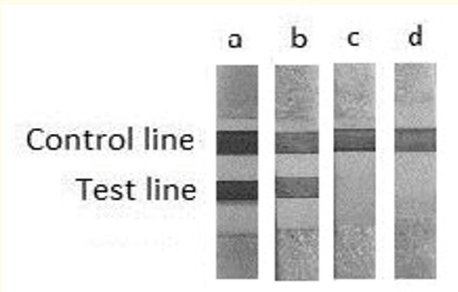

Figure 3: (A) Photo image of strips with varying concentrations of synthetic target DNA in the sample. (a) 0 nM (-), (b) 1 nM (+++), (c) 0.5 nM (++), (d) 0.25 nM (++), (e) 0.125 nM (-), (f) 0.06 nM (-). (B) Intensity of the bands analyzed by GrafphPad-Prism®, Model: Sigmoidal 4PL, R square: 0.9906; Top: 270245; Bottom: -418; IC50: 1.179. (C) Photo image of strips when PCR mix was used as the sample (a) synthetic target DNA used as positive control of biosensor 100 nM in 100 µL 4X SSC buffer, (b) 262 nM PCR product using L. monocytogenes DNA as template 25 µL PCR amplification mix + denaturation at 95ºC 1 min + 75 µL 4X SSC buffer, (c) PCR product using L. innocua DNA as template 25 µL PCR amplification mix + denaturation at 95 ºC 1 min + 75 µL 4X SSC buffer and (d) PCR negative control without DNA 25 µL PCR amplification mix + denaturation at 95 ºC 1 min + 75 µL 4X SSC buffer.

Conclusion

In summary, we have successfully generated a biosensor based on lateral flow oligonucleotide detection, rapid and sensitive. Three main physical parameters, membrane type, gold nanoparticles size and amount of reporter probe were evaluated. The migration speed clearly affects the response, we found that with the slower flow membrane, the assay resulted in a longer time assay (10 minutes vs 5 minutes) but the response signals were higher, and the bands were better defined. The sensitivity of the assay was higher when 1 OD of reporter probe was immobilized on any size of gold nanoparticles. We used *L. monocytogenes* as model to evaluate strips prepared with 1 OD of reporter DNAprobe_3 conjugated to 12 nm nanoparticles and UniStart CN 140 membrane. A well-defined typifying fragment of *L. monocytogenes* was used to evaluate this lateral flow assay. We found that the sensitivity was 25 fmol when synthetic single stranded DNA was used as sample, in only 10 min assay time. When the biosensor was evaluated against real samples after amplification of the amplicon by PCR, the assay performed specifically; no cross-reactivity with *L innocua* or PCR control mix was observed. It is to be noted, that PCR product can be applied directly onto the sample pad without need of pre-incubation or purification step, minimizing assay time. The screening of food borne pathogens in real samples requires culturing in selective media followed by identification and quantification. Reported identification and quantification methods are time consuming or need complex equipment (e.g. Real-Time PCR termocycler, PFGE, MALDI-TOF). LFA type assay such as the assay developed here, are rapid and easy to use after the enrichment or amplification step, without the need of complex equipment at the point of care, in clinical and non-clinical applications. The methodology described in this work can be easily applied to any pathogen, as long as, it´s typifying sequence is known.

Acknowledgements

Funding by the Parque Científico y Tecnológico de Pando, Uruguay is gratefully acknowledged.

Conflict of Interest

There is no conflict of interest between authors.

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