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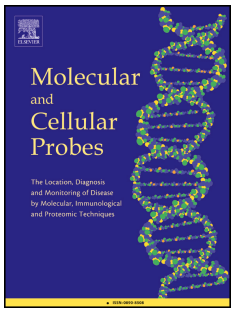

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Original Research Paper

Nanoparticle-Based Lateral Flow Biosensor for Visual Detection of Fish Nervous Necrosis Virus Amplification Products

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Abstract: Lateral flow paper biosensors are an attractive analytical platform for detection of human and veterinary disease pathogens because they are optimal for accurate, rapid and sensitive analysis in research laboratory setups, as well as field analysis. Since diseases of viral etiology have been wreaking havoc in aquaculture industry, as well as the environment, the present study aims at the development of a gold nanoparticle-based biosensor for fish nervous necrosis virus (Nodavirus) nucleic acids detection. Total viral RNA, isolated from fish samples was subjected to reverse transcription PCR amplification. The PCR products were mixed with a specific oligonucleotide probe and applied next to oligonucleotide conjugated Au NPs. A red test line was formed when nodavirus product was present. The visual detection of the RT-PCR product was completed within 20 min. Following optimization, the biosensor was able to visually detect 270 pg of nodavirus

initial total RNA. The present study describes a simple, accurate, robust and low cost method for nodavirus detection in biological samples. Apart contribution on basic research, the proposed biosensor offers great potential for commercial kit development for use on the site of fish culture by fish farmers. This fact will have great impact on environmental safety and disease monitoring without time consuming and costly procedures.

Keywords: Lateral flow strip biosensor; gold nanoparticles; nervous necrosis virus; Nodavirus; RT-PCR; *Dicentrarchus labrax*

1. Introduction

Viral nervous necrosis (VNN), also named vacuolating encephalopathy and retinopathy (VER) or encephalomyelitis, is a disease that causes high mortalities (80-100%) in several marine fish species in Europe, America, Japan, Southeast Asia, and Australia [1]. In recent years, VNN has attracted much attention because of its ecological and economic impacts on the aquaculture industry. Especially in the Mediterranean area, VNN has emerged as a major problem for aquaculture facilities, as well as wild marine species, since it has no effective vaccine or treatment [2,3]. The causal agent of the disease is the nervous necrosis virus (NNV), also known as nodavirus, which has been reported worldwide in at least 30 different fish species, including groupers, sea bass and Atlantic halibut, indicating a wide host range [1,4-7].

Fish nodavirus (genus: *Betanodavirus*, family: *Nodaviridae*) is an icosahedral, non-enveloped virus with diameter of ~25 nm [1]. Its genome is composed of two, single-stranded, positive-sense RNA molecules of 3.1 kb (RNA 1) and 1.4 kb (RNA 2). RNA 1 directs the synthesis of the RNA-dependent RNA polymerase (100 kDa) [8] and RNA 2 encodes the synthesis of the coat protein of the virus (42 kDa) [9]. RNA 3 is a subgenomic transcript of the RNA1 segment which contains an open reading frame for the B2 protein [10]. Nodavirus has been classified in four genotypes: striped jack NNV

(SJNNV), red-spotted grouper NNV (RGNNV), tiger puffer NNV (TPNNV) and barfin flounder NNV (BFNNV). Fish nodaviruses are quite different from insect nodaviruses (*alphanodaviruses*) [11]. A nodavirus found only in shrimps and prawns, *Macrobrachium rosenbergii* nodavirus has been identified. Its sequence could not be assigned to either of the existing genera and it has been proposed to be classified as *Gammanodavirus* [12].

Fish nodavirus has been detected and analyzed with several methods including light- and electron-microscopy [1,13], immunofluorescence antibody test (IFAT) [14], enzyme-linked immunoassay (ELISA) [15] and *in situ* hybridization [16]. However, polymerase chain reaction (PCR) [9] and reverse transcriptase PCR (RT-PCR) [17] have become the main diagnostic methods for fish nodaviruses, as they can detect tiny quantities of viral RNA in any tissue. Analysis of PCR products (amplicons) is, usually, carried out by agarose gel electrophoresis, followed by ethidium bromide staining. Several methodologies for rapid and accurate nodavirus detection have been developed recently, including: real-time quantitative PCR [18]; loop-mediated-isothermal-amplification (LAMP) [19] and real-time LAMP [20] assays; an immunomagnetic reduction (IMR) assay which employs magnetic nanoparticles coated with dextran and antibodies [21]; a microfluidic LAMP system, needing slab-electrophoresis for products detection [22]; a RT-PCR microfluidic chip with laser-induced fluorescence technology on the capillary electrophoresis module of the chip [23]; and an integrated microfluidic chip with molecular beacons and nucleic acid sequence-specific captured probes conjugated to magnetic beads [24]. Even though each mentioned methodology has several advantages, they require expensive and specialized instrumentation, highly-trained personnel or they are time-consuming and cumbersome. Especially the methods that utilize gel electrophoresis for products detection are based on products size detection, which might result in false positives, and are using the highly mutagenic ethidium bromide.

Paper-based sensing has emerged as an attractive analytical platform. Lateral flow paper biosensors (LFB) are optimal for accurate, rapid and sensitive analysis in research laboratory setups, but they have the potential to be used for field analysis. Especially the use of functionalized nanoparticles, as

recognition labels has accelerated nucleic acids/ proteins sensing by LFBs, by increasing their sensitivity and specificity. In general, lateral flow biosensors are prefabricated strips of materials containing dry reagents that are activated by applying the fluid sample. They are designed for disposable single use and for applications where an on/off signal is sufficient [25]. LFBs are considered one of the most promising technologies owing to their simplicity, rapid analysis, low cost, high sensitivity and specificity. Several lateral flow biosensors have been developed to detect many analytes such as DNA, mRNA, miRNA, proteins, biological agents and chemical contaminants [26].

In that aspect, the present study aims at the development of a gold nanoparticle-based LFB for nodavirus nucleic acids detection. Specifically, in order to increase the detection accuracy, simplify and speed up the total time for PCR-based analysis, we propose a paper lateral flow biosensor which has been evaluated in applications such as hepatitis C virus detection [27]; genetically modified organisms (GMO) detection [28]; bacterial infections detection [29] and single nucleotide polymorphisms (SNPs) genotyping [30,31]. In brief, total viral RNA, isolated from fish samples was subjected to reverse transcription PCR amplification. The PCR products were mixed with specific oligonucleotide probe and applied next to oligonucleotide conjugated gold nanoparticles (Au NPs). A red test line was formed when nodavirus product was present. The visual detection of the RT-PCR product was completed within 20 min. Detection parameters were optimized and the proposed LFB discriminate NNV infected from non-infected samples successfully.

2. Experimental Section

2.1. Oligonucleotides

All oligonucleotides used in this study were synthesized by VBC-Biotech (Vienna, Austria). A 5′-thiolated $(dT)_{30}$ oligonucleotide (SH-dT_{30}: 5′-SH-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′) was used for conjugation on gold nanoparticles. A $(dA)_{20}$ oligonucleotide (dA_{20}: 5′-AAAAAAAAAAAAAAAAAAAA-3′) was used for construction of the control zone of the biosensor. The 20-mer 5′-TAAGAAATTGGCAAAACCCG-3′ was labeled at the 5′-end with biotin and used as

an upstream primer (UpNdv_B) for PCR of nodavirus coat protein cDNA. The 20-mer 5′-TATCCGTCTGTTCCTGTCCC-3′ was used as downstream primer (DpNdv). The 20-mer 5′-CCTTAGACACAGGTGCGTCA-3′ was used as the nodavirus-specific probe (Probe_Ndv). The oligonucleotide primers and probe were designed based on the published sequence for nodavirus coat protein gene (GenBank accession number: Y08700.1). Finally, B-dA_{20} (5′-B-AAAAAAAAAAAAAAAAAAAA-3′) reference oligonucleotide was used as control for biosensor construction. : ~~B-dT_{20}, 5′-B-TTTTTTTTTTTTTTTTTTTT-3′ and~~.

2.2. Tailing of Oligonucleotide Probes with dTTP or dATP

The 5′-SH modified $(dT)_{30}$ oligonucleotide was tailed enzymatically with dTTP, whereas the $(dA)_{20}$ probe, the biotinylated control oligonucleotide B-dA_{20}, and the nodavirus-specific probe (Probe_Ndv) were tailed with dATP. The reactions were performed in a total volume of 20 µL, containing 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate (pH 7.9), 0.25 mM $CoCl_2$, 3.5 mM dTTP or dATP (2 mM of dATP for probe Ndv), 10 units of terminal deoxynucleotidyl transferase (TdT; New England Biolabs, Ipswich, MA, USA), and 700 pmol of oligonucleotide (400 pmol for probe_Ndv). The reactions were carried out at 37 ºC for 60 min and terminated by heating to 70 ºC for 10 min. After completion of the tailing reaction the poly(dA)-tailed Ndv probe was mixed with 1.5 µL of 40 mM N-methylmaleimide (Sigma-Aldrich, Steinhem, Germany) solution in dimethyl-sulfoxide (DMSO, 2.7 mM final concentration). The tailed probes were stored at -20 ºC.

2.3. Preparation of Oligonucleotide Conjugated Gold Nanoparticles

Gold nanoparticles (40 nm, 7.2×10^{10} particles/mL; Sigma-Aldrich, Steinhem, Germany) were modified with oligonucleotide addition, through thiol group conjugation [27]. The reactions were carried out by adding 75 pmol of tailed $(dT)_{30}$ oligonucleotide and 0.8 µL of absolute pyridine to 1 mL

of the gold nanoparticle solution (~0.12 pmol). The mixture was incubated at 4 ºC, for 24 h. Subsequently, the solution was subjected to "aging" by the addition of NaCl up to a concentration of 90 mM. The solution was allowed to stand for another 24 h at 4 ºC, and the excess of reagents was removed by centrifugation at 1,300×g for 30 min. The supernatant was discarded, and the pellet was redispersed in 100 µL of an aqueous solution containing 30% sucrose, 0.25% Tween-20, 0.25% sodium dodecyl sulfate (SDS), and 45 mM NaCl, by vortexing and brief sonication.

2.4. Preparation of the Lateral Flow Biosensors

The dry reagent lateral flow biosensors (4×60 mm) consisted of a cellulose immersion pad, a glass-fiber conjugate pad, a nitrocellulose diagnostic membrane, and a cellulose absorbent pad. The parts were assembled on a plastic adhesive backing as follows: The diagnostic membrane (HF240MC100, 25 mm in length; Millipore, Billerica, MA, USA) was placed on center of a laminated card by the manufacturer. The conjugate pad (GFCP000800, 8 mm; Millipore, Billerica, MA, USA) was then placed below the membrane, the immersion pad (CFSPOO1700, 17 mm; Millipore, Billerica, MA, USA) was placed below the conjugate pad, and the absorbent pad (same as immersion pad) was positioned above the membrane. Each part overlapped 2 mm to ensure that the solution could migrate through the biosensor. The TLC applicator, Linomat 5 and WinCats software (Camag, Muttenz, Switzerland), were employed to construct the test zone and the control zone by loading streptavidin (SA) from *Streptomyces avidinii*, and poly(dA), respectively, on the membrane. In details, a solution containing 4 g/L SA (Sigma-Aldrich, Steinhem, Germany), 150 mL/L methanol, and 20 g/L sucrose was loaded at a density of 1.6 µg/4 mm membrane. A solution containing 4 µM poly(dA)-tailed probe, 500 mL/L methanol, and 20 g/L sucrose was loaded at a density of 2.4 pmol/4 mm membrane. The membrane was then dried at 80 ºC for 1 hr. LFBs with a 4-mm width were cut by using a Guillotine cutting and stored dry at ambient temperature.

2.5. Fish Samples

The European sea bass (*Dicentrarchus (D.) labrax*) with or without VNN clinical signs were collected from sea-cage fish farms in Epidavros (Saronikos Gulf), Astakos (Ionian sea) and Aliveri (Euboean Gulf) regions, covering three of the major aquaculture sites in Greece. Asymptomatic fish reared for 8 months into experimental land facilities of the Hellenic Centre for Marine Research (Athens, Greece), were used as healthy negative control fishes. Samples were processed using aseptic technique to isolate dissected brain and retinas for RNA extraction. Samples were transferred in sterile polypropylene tubes and stored at −80 ºC, until use.

2.6. RNA Extraction

Total RNA was extracted from the fish eyes using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration and purity were determined by measuring the absorbance at 260 nm (A_{260}) in the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). The purified aliquots had concentrations in the range of 100 – 180 ng/mL of total RNA in RNase-free water. The A_{260}/A_{280} ratios of the purified aliquots were 2.04-2.08, indicating that the isolated total RNA was efficiently purified for further use.

2.7. RT-PCR of NNV RNA from Fish Samples

In order to reverse transcribe (RT) the NNV total RNA, the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) was used. The RT reaction consisted of 2.5 μM oligonucleotide dT_{20}, 0.5 mM dNTPs (dNTPs: dATP, dTTP, dCTP, dGTP; HT Biotechnology, Cambridge, UK), 100 ng of purified total RNA and RNase-free H_2O. The mixture was heated to 65 ºC for 5 min, and quickly chilled on ice (0 ºC, 1 min). First-strand buffer (1x), Dithiothreitol (0.1 M) and RNase OUT RNase inhibitor (40 u; Invitrogen, Carlsbad, CA) were added to the mixture, which was incubated at 42 ºC for

2 min. Immediately, 200 units of SSII RT were added and the mixture was incubated at 42 ºC for 50 min. Finally, the mixture was heated at 70 ºC for 15 min, in order to deactivate the enzyme and the produced cDNA was stored at -20 ºC.

The produced cDNA was subjected to PCR. The reaction mixture (25 µL) contained 1x GoTaq Flexi Buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM each of the upstream and downstream primers, 2 µL of cDNA, and 0.5 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA). The cycling conditions were 95 ºC for 5 min, followed by 35 cycles at 95 ºC, 30 s; 60 ºC, 60 s; and 72 ºC, 60 s. At the end of the cycling, the mixture was incubated at 72 ºC for 7 min and cooled down to 4 ºC. PCRs were performed in GeneAmp PCR System 9700 cycler (Applied Biosystems, New York, USA). Negative controls (containing water instead of DNA) were included in each series of PCRs to confirm the absence of contamination. The PCR products were visualized by 2% agarose gel electrophoresis and quantified based on the φX174 DNA *HaeIII* digest DNA molecular weight marker (HT Biotechnology, Cambridge, UK). The Sub-Cell GT agarose gel electrophoresis system was from Bio-Rad (New York, USA). The digital camera and UV transilluminator, AlphaEase FC Imaging System, was from Alpha Innotech (California, USA) and the gel images were analyzed with AlphaImager software and ImageJ software [32].

2.8. Lateral Flow Biosensor Detection Assay of Nodavirus Amplification Products

For the detection of NNV PCR products, a 5-µL aliquot of PCR solution was mixed with 1 µL of 0.9 M NaCl, 0.5 pmol of dATP-tailed NNV probe and ddH_2O in a total volume of 10 µL. The mixture was heated at 95 ºC for 3 min and then hybridization was allowed to proceed for 10 min at 37 ºC. The above mixture was applied to the conjugation pad next to the poly(dT)-functionalized gold nanoparticles. The immersion pad of the biosensor was then dipped into 250 µL of the developing solution. The developing solution contained 40 mL/L glycerol, and 10 g/L SDS in phosphate-buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 10 mM sodium phosphate and 1.7 mM potassium phosphate,

pH 7.4). The visual detection of was completed within 20 min. Longer times did not affect the assay results. After completion of the assay, the LFBs were scanned with a desktop scanner (HP Scanjet G4050, HP, California, USA) and the band densities were quantified with ImageJ software [32].

3. Results and Discussion

3.1. Assay Principle

The principle of the nanoparticle-based lateral flow biosensor is depicted in figure 1. After the total RNA isolation from fish samples, a RT reaction is performed. The produced cDNA is amplified and end-labeled with biotin during PCR, by using an upstream primer labelled with biotin. The PCR product is mixed with a nodavirus specific poly(dA)-tailed oligonucleotide probe and hybridization results on a poly(dA) probe – sample complex. The hybridized complex is applied on the conjugate pad next to Au NPs that are surface-functionalized with poly(dT). Subsequently, the LFB is immersed in the developing solution which migrates along the biosensor by capillary action. As a result, the poly(dT) functionalized Au NPs move towards the hybridized complex, interact and hybridize with the poly(dA) probe – sample complex. The hybrids are finally captured from immobilized streptavidin at the test zone of the biosensor. The accumulation of Au NPs generates a characteristic red line at the test zone since gold nanoparticles have an absorbance peak at 520 nm due to plasmon resonance. The excess nanoparticles are captured from immobilized oligo(dA) strands at the control zone of the LFB generating a second red line that confirms the proper function of the biosensor. In the absence of target DNA, no red band is observed at the test zone.

3.2. Optimization of the lateral flow biosensor with a reference oligonucleotide

Since the accumulation of Au NPs on the test and control zones could affect the signal formation, two major factors were investigated by comparing the analytical performances of the LFBs on specifically detecting a reference oligonucleotide. The studied factors were the amount of Au NPs and the composition of the developing solution. The reference oligonucleotide B-dA_{20}, tailed with dATP,

had a biotin moiety in the 5' end, in order to interact with streptavidin in the biosensors' test zone, and a poly(dA) tail in the 3' end, to interact with poly(dT) Au NPs.

3.2.1. Effect of the Au NPs amount

The amount of gold nanoparticles loaded on the conjugate pad was determined by the nominal concentration of the nanoparticles (7.2×10^{10} particles/mL) and the respective volume of solution applied on the conjugate pad. The target mix consisted of 500 fmol B-dA_{20}, 90 mM NaCl to facilitate hybridization, and ddH_2O. The amount of the nanoparticles was optimized in the range of 3 – 15 fmol per LFB. It was found that 12 fmol gave strong color intensities in both the test and control zones with no background (figure 2A). Even though higher nanoparticles amount increase the band intensities, the background signal also increased, as it can been seen in the LFB images. Therefore, the use of 12 fmol of Au NPs conjugates, corresponding to 10 μL of the prepared solution, was adopted in the following experiments.

3.2.2. Effect of the developing solution composition

The composition of the developing solution affects critically the performance of the DNA hybridization on the LFB. The developing solution should allow the sample mixture and Au nanoparticles to migrate at a flow rate that both provides a sufficient time for the hybridization reaction to occur and also ensures a rapid test, and to prevent the nonspecific binding of the nanoparticles on the biosensor membrane [27]. The addition of glycerol results in increased viscosity of the developing solution and causes a decrease of the flow rate. Various surfactants (e.g. SDS, Tween-20) were added in order to facilitate the movement of the nanoparticles from the conjugate pad. Several formulations were tested, as we observe in figure 2B. In details, the solutions that were tested consisted of 1% SDS (1%S), 2 % glycerol and 1% SDS (2%G_1%S), 4 % glycerol and 1% SDS (4%G_1%S), 4% glycerol, 1% SDS and 0.1% Tween-20 (4%G_1%S_0.1%T), 4% glycerol, 1% SDS and 1% Tween-20 (4%G_1%S_1%T), 6 % glycerol and 1% SDS (6%G_1%S), 6% glycerol, 1% SDS and 1% Tween-20

(6%G_1%S_1%T), 8% glycerol and 1% SDS (8%G_1%S), 8% glycerol, 1% SDS and 1% Tween-20 (8%G_1%S_1%T), all in PBS buffer. The optimum combination for clear bands, rapid assay times, and background elimination was attained with use of 6% glycerol, 1% SDS and 1% Tween-20.

3.3 Optimization of the lateral flow biosensor assay with nodavirus amplification products

In order to achieve the optimum results for nodavirus detection in samples, two parameters of the poly(dA) probe – sample complex formation assay were studied: the hybridization temperature and the amount of the specific oligonucleotide probe in hybridization mixture.

3.3.1. Effect of the hybridization temperature

The effect of target PCR product and specific oligonucleotide probe hybridization temperature was studied in the range of 25 – 55 ºC. The results are presented in figure 3A. The density of the test zone reaches the optimum at 37 ºC and decreases in higher temperatures, probably due to hybridization inhibition. Therefore, all subsequent hybridizations were performed at 37 ºC.

3.3.2. Effect of the hybridization temperature

The influence of the amount of oligonucleotide probe in the hybridization mixture was studied in the range of 0.25 – 2.5 pmol/ 1 pmol of target (figure 3B). The density of the test zone was optimum with 0.5 pmol probe and decreased with higher amounts of probe. This is because, at high levels, the amount of poly(dA) oligonucleotide probe exceeds the binding capacity of the poly(dT)-functionalized nanoparticles. As a consequence, poly(dA) oligonucleotide that is hybridized to the target sequence competes with the free (unhybridized) poly(dA) oligo for binding to limited poly(dT) strands on the nanoparticles. The higher the amount of poly(dA) oligo used, the larger the fraction of nanoparticles that bind to the free probe. These nanoparticles then migrate along the entire length of the biosensor

without being captured from immobilized streptavidin and poly(dA). As a result, the red bands of both the test zone and the control zone become fainter [27].

3.4. Analytical characteristics of nodavirus LFB

3.4.1. Nodavirus LFB detectability

To assess the biosensor applicability, sample solutions containing different concentrations of target PCR products were tested under the optimized experimental conditions. Specifically, various amounts of PCR products (107 bp) ranging from 0 to 1000 fmol, corresponding to initial total RNA amount in the range of 135 pg to 1 ng per reaction, were applied on LFBs. The concentration of each PCR product was determined by agarose gel electrophoresis, ethidium bromide staining and densitometry with φX174 – *HaeIII* digest DNA fragments as calibrators. The respective LFBs are presented in figure 4. As low as 270 pg of initial total RNA (250 fmol of target DNA) was detectable with the naked eye on the biosensor. Densitometrically, 135 pg of initial total RNA (125 fmol of target DNA) were corresponded to a positive signal. To define a positive result, we used the signal/noise (S/N) ratio (S/N = 3) as reference. Signal is the optical intensity of the test line with detected sample and noise is the intensity of the test line with 1×GoTaq Flexi Buffer.

The same amount of products was applied on agarose gel and visualized with ethidium bromide staining. As we observe in figure 4c, 135 pg of initial total RNA are faintly visible on the gel revealing that the proposed biosensor could achieve comparable sensitivity.

3.4.2. Nodavirus LFB reproducibility

Since the signal reproducibility is one of the most important parameter to evaluate the biosensor, it was assessed with i) the reference oligonucleotide, and ii) nodavirus amplification products. Initially, the reproducibility was determined by analyzing 500 fmol of B-dA_{20}. Five biosensors were tested with or without the reference oligonucleotide for study of the test or the control zone, respectively. The five

biosensors were produced in different batches. The results are presented in figure 5A. Densitometric analysis of the test zones of the strips gave coefficient of variation (CV) of 11.1%, while analysis of the control zones resulted in 5.1% CV.

Subsequently, the reproducibility was studied in real analysis conditions, where samples of 5 μL of PCR product hybridized with 0.5 pmol of nodavirus probe, were loaded on 5 LFBs. The assay results are presented in figure 5B. The CVs for test and control zones were 4% and 7%, respectively, indicating excellent reproducibility.

3.4.3 Nodavirus LFB specificity

The specificity of the LFB for nodavirus detection was determined by checking the cross-reactivity of the assay with RNA extracted from eye tissues of non-infected fish. Amplicons were detected only for the verified infected samples (figure 6).

3.5. Nodavirus detection on D. labrax samples with the LFB assay

The nodavirus biosensor was used to detect nodavirus in *D. labrax* samples with the optimized reaction conditions. Ten indicative infected (positive) and healthy (negative) *D. labrax* samples were analyzed, as shown in figure 6. The non-infected samples were obtained from experimental land facilities in a controlled environment, in order to assure that no infection had occurred. Two samples were originated form Epidavros area (S5-6), 3 samples were from Aliveri region (S7-9) and 1 sample was collected from Astakos (S10). In each run a negative control, containing 1× GoTaq Flexi Buffer instead of a PCR product, and a positive control, containing 500 fmol of B-dA_{20}, were included. None of the non-infected samples showed any signal in the test zone. All infected samples were positive with varying signal intensities, corresponding to different amounts of nodavirus in the sample.

4. Conclusions

Rapid and accurate detection of nodavirus would enable the early quarantine of infected fishes in order to protect the aquaculture breeding facilities. Mostly due to its economic impact to aquaculture industry, methods for efficient identification of infectious diseases have attracted considerable interest in recent years. As a step forward to rapid analysis of virus amplification products we developed a nanoparticle-based paper lateral flow biosensor for simple and specific visual detection of nodavirus RT-PCR products in biological samples. The target nodavirus amplification product could be easily detected by observing the test zone formation, resulting in an intense, red colored signal. Under optimal conditions, 270 pg of initial total RNA were detectable with the naked eye within 20 min. Alternatively, the biosensor scanning with a simple desktop scanner or a smart phone allowed detection of 135 pg of initial total RNA. The total assay starting from RNA extraction, performing RT-PCR and detecting the products with the proposed LFB was completed in less than 4 h.

Compared to traditional methods (i.e. gel electrophoresis), the proposed method offers a simple, fast, selective and sensitive approach to detect nodavirus PCR products without expensive instrumentation. Detection of the PCR products is accomplished by target-probe hybridization providing sequence confirmation; as opposed to electrophoresis which give results based only on the size of the amplified fragments. The analysis/detection time with the proposed LFB is 20 min, compared to approximately 2 h when the agarose gel methodology is employed (gel preparation, sample loading and running). The pre-hybridized sample can be applied directly on the biosensor and a small amount of developing solution is needed, reducing thus the wastes amount. Also, as mentioned above, the proposed biosensor is as sensitive as the traditional detection method (figure 4c). Finally, the proposed LFB utilizes a small amount of gold nanoparticles for PCR products visualization, eliminating the need for highly toxic/ mutagenic ethidium bromide, which is routinely used in agarose gel electrophoresis.

Even though lateral flow biosensors based on immunochromatography have been introduced for several fish pathogens analysis [33], nucleic acid LFBs are on high demand today. To our knowledge, there has been only one other published effort to apply a generic lateral flow dipstick for

Macrobrachium rosenbergii nodavirus detection [34]. In that process, generic LFD strips (Milenia® GenLine HybriDetect, Milenia Biotec GmbH, Germany) were used to detect biotin labeled amplicons hybridized with an FITC-labeled DNA probe linked, in turn, to a gold-labeled anti-FITC antibody. The same generic LFD strips has been applied on *Taura* syndrome virus [35], hepatopancreatic parvovirus [36], infectious myonecrosis virus [37], infectious hypodermal and hematopoietic necrosis virus [38], yellow head virus [39], *Laem-Singh* virus [40], *Penaeus monodon* nucleopolyhedrovirus or monodon baculovirus [41] and white spot syndrome virus [42] for shrimp viruses detection; as well as fish infectious spleen and kidney necrosis virus detection [43] and cyprinid herpes virus-3 or koi herpes virus [44]. In all cases, loop mediated isothermal amplification (LAMP) has been utilized for nucleic acid amplification, instead of traditional PCR, which is used in the proposed method. It has been reported that the RT-LAMP method, used for NNV detection, is more sensitive than the RT-PCR method [22] and, even though, a direct comparison of sensitivity for the applied methodologies wouldn't be accurate, it should be noted that the proposed method is, also, capable to detect picograms of initial total RNA.

Several techniques for fish nodavirus detection have been developed. The assay time (RT reaction, amplification step and detection) and the sensitivity for each method are summarized in table I. The assay time for the proposed method is ~ 3h while 135 pg of total RNA (per 20 μL reaction) can be detected, by using a standard PCR machine and the low cost LFB. The most common approach consists of conventional PCR [7,9,17,45-48] or nested PCR [17,45,46,49] with agarose gel electrophoresis detection. Real-time PCR [18,47,48,50-53] is less time consuming but requires more expensive instrumentation and/or reaction reagents. LAMP amplification methods with gel electrophoresis detection [19,45,46] or real time [20] format have also been applied on nodavirus detection. Real time nucleic acid sequence based amplification (NASBA) [54], RT-PCR combined with dot-plot detection [7], a LAMP-based microfluidic devise [22], a RT-PCR microfluidic devise with capillary electrophoresis detection (CE) [23] and a microfluidic device based on molecular beacons and magnetic separation [24] have, also, been utilized for nodavirus analysis. Based on table I,

we can assume that the proposed methodology is less time consuming than most of the aforementioned techniques, except the real-time based methodologies and the microfluidic devises, which, however, have higher costs and/ or require sophisticated instrumentation. In our opinion the methods' sensitivity comparison cannot be accurate since the methodologies for sensitivity determination are quite variable amongst the published results, arising from various research groups. In most cases the sensitivity is determined with virus-containing cell cultures supernatants. In an effort to be more realistic, we studied the LFBs' sensitivity in a more complex sample type, consisting of total RNA isolated from infected tissue. The research groups that follow the same approach express the sensitivity with a dilution factor, making thus direct correlations difficult. Empirically, the proposed methods' detection limit, which is at the pg level of initial total RNA, is considered sufficient for routine analysis. Efforts on improving sensitivity are ongoing for the proposed LFB.

The proposed LFB is based on nucleic acids for gold nanoparticles conjugation as well as control zone fabrication, eliminating the need for use of antibodies. Therefore it has higher stability and no need for special storage. The cost of the assay in terms of reagents and the biosensor is less than 3 €. The biosensor allows visual detection of nodavirus amplification products within minutes without the need of any instruments. ~~Detection of the PCR products is accomplished by target-probe hybridization providing sequence conformation as opposed to electrophoresis that gives only the size of the amplified fragments.~~ The performance of the proposed LFB was assessed by analyzing nodavirus PCR products but it can be applied to other fish pathogens analysis, by designing appropriate primers and probes and using the same biosensor. In our opinion, the proposed assay is extremely useful to small scale research labs and aquatic industries quality control facilities.

Ongoing studies include the development of an isothermal method for nodavirus nucleic acids amplification, which combined with the proposed biosensor will allow easiest nodavirus detection on the site of fish culture by fish farmers, and improvements in the sensitivity of the LFB by using gold nanoparticles dual labels and other LFB signal enhancement systems.

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Author Contributions

D.K.T. conceived and designed the experiments; D.K.T and M.M. performed the experiments; D.K.T. and E.K. analyzed the data; D.K.T. wrote the paper; M.M. and E.K. proof-read the manuscript; D.K.T. and E.K. supervised the work. All authors have approved the present article.

Conflicts of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results

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Figure Captions

Figure 1. Principle of the nanoparticle-based lateral flow biosensor for nucleic acid detection. **a.** Side view of the lateral flow biosensor. IP: immersion pad; CP: conjugation pad; M: diagnostic membrane; AP: absorbent pad. A test zone (streptavidin-SA) and a control zone (poly(dA)) have been immobilized on the biosensors' diagnostic membrane. **b.** The sample (S) contains the target analyte (PCR product) hybridized with a poly(dA)-tailed oligonucleotide probe and biotin (B) moieties and is applied on the conjugation pad next to by poly(dT) functionalized gold nanoparticles (Au). **c.** The biosensor is dipped on the developing buffer, the sample and the nanoparticles are captured on the test zone and the positive signal is visualized, as a red zone. The excess nanoparticles bind to poly(dA) at the control zone of the biosensor. The assay components are not in scale.

Figure 2. a. Effect of the Au NPs amount loaded on the conjugate pad, on the LFB test zone signal intensity. **b.** Effect of the developing solution composition on the LFB test zone signal intensity. G: Glycerol; S: SDS; T: Tween-20.

Figure 3. a. Effect of the target PCR product and specific oligonucleotide probe hybridization temperature on the LFB test zone signal intensity. **b**. Effect of the oligonucleotide probe amount, in the hybridization mixture, on the LFB test zone signal intensity.

Figure 4. a. Typical images of the LFBs after applying different amounts of nodavirus initial total RNA. **b**. Graph of the intensities of the LFBs test zones versus the nodavirus initial total RNA amounts. **c.** Agarose gel (2%) electrophoresis of the above target DNA followed by ethidium bromide staining. Lane M: φX174 DNA *HaeIII* digest DNA molecular weight marker; Lane N: PCR negative control; Lanes 1, 2, 3 and 4 contain 135, 270, 500 and 1000 pg per lane of nodavirus initial total RNA, respectively.

Figure 5. Reproducibility study of **a**. LFB test zone with reference oligonucleotide B-dA_{20} as target (CV: 11.1%, n = 5). **b**. LFB control zone with reference oligonucleotide B-dA_{20} as target (CV: 5.1%, n = 5). **c**. LFB test zone with nodavirus PCR product as target (CV: 4%, n = 5). **d**. LFB control zone with nodavirus PCR product as target (CV: 7%, n = 5).

Figure 6. Visual detection of nodavirus amplification products in infected and non-infected *D. labrax* samples with lateral flow biosensors. NC: Negative control; PC: Positive control; N1-4: Non infected samples; S5-10: Infected samples.

Table I. Comparison of fish nodavirus detection methodologies.

Table I. Comparison of fish nodavirus detection methodologies.

	Assay Time*	Sensitivity		Reference
		Cultured virus	Infected tissue	
RT-PCR/ Electrophoresis	~ 4.5 h	2.58×10^{2} $TCID_{50}$ / mL		[45]
	~ 4 h	1–10 $TCID_{50}$ / mL		[7]
	~ 4 h		10^{-3} dilution total RNA	[46]
	~ 5.4 h	4.9 fg RNA/ µL		[47]
	~ 4 h	$10^{1.8}$ $TCID_{50}$ / mL		[48]
	~ 4 h	10^{-4} dilution		[17]
	~ 4 h		10 fg/ viral RNA	[9]
RT-PCR/ dot-plot	~ 1d	10^{-1}–1 $TCID_{50}$ / mL		[7]
RT-PCR Microfluidic/ CE	~ 2 h		3 copies/ mL total RNA	[23]
RT-PCR/ LFB	~ 3 h		135 pg total RNA	Present study
Nested PCR / Electrophoresis	~ 5.5 h	2.58 $TCID_{50}$ / mL		[45]
	~ 4 h		10^{-5} dilution total RNA	[46]
	~ 5.5 h	10 -100 $TCID_{50}$ / mL		[49]
	~ 5 h	10^{-4} dilution		[17]
LAMP amplification/ Electrophoresis	~ 3 h	2.58×10^{-2} $TCID_{50}$ / mL		[45]
	~ 2-4 h		10^{-8} dilution total RNA	[19]
	~ 3 h		10^{-6} dilution total RNA	[46]
LAMP Microfluidic/ Electrophoresis	~ 4.5 h		10–100 fg total RNA	[22]
Real-time PCR	~ 2 h		10^{-8} dilution total RNA	[50]
	~ 2 h	1.9×10^{2}copies (cDNA)		[18]
	30 min	30 $TCID_{50}$ / mL		[51]
	~ 3 h	10 $TCID_{50}$ / mL		[52]
	~ 1 h	0.04 - 0.4 $TCID_{50}$ / mL		[53]
	~ 2 h	19.5 fg RNA/ µL		[47]
	40 min	$10^{0.8}$ $TCID_{50}$ / mL		[48]
Real-time NASBA	~ 2 h	1.0 - 0.1 $TCID_{50}$ / mL		[54]
Real-time LAMP	~ 2 h	4×10^{-11} copies/µL plasmid		[20]
Molecular beacon/ Magnetic / Microfluidic	~30 min	20ng/mL plasmid		[24]