Detection of isothermally amplified ostreid herpesvirus 1 DNA in Pacific oyster (*Crassostrea gigas*) using a miniaturised electrochemical biosensor

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Abstract

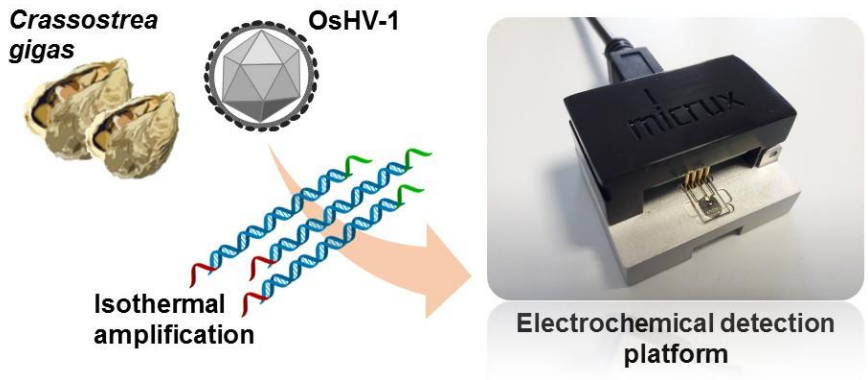
Given the threat that ostreid herpesvirus 1 (OsHV-1) poses to shellfish aquaculture, the need for rapid, user-friendly and cost-effective methods to detect this marine pathogen and minimise its impact is evident. In this work, an electrochemical biosensor for the detection of OsHV-1 based on isothermal recombinase polymerase amplification (RPA) was developed. The system was first tested and optimised on maleimide microtitre plates as a proof-of-concept, before being implemented on miniaturised gold electrodes. Amperometric detection of the isothermally amplified product was achieved through a sandwich hybridisation assay with an immobilised thiolated capture probe and a horseradish peroxidase (HRP)-labelled reporter probe. Calibration curves were constructed using PCR-amplified OsHV-1 DNA, achieving a limit of detection of 207 OsHV-1 target copies. The biosensor was applied to the analysis of 16 oyster samples from an infectivity experiment, and results were compared with those obtained by qPCR analysis, showing a strong degree of correlation (r = 0.988). The simplicity, rapidity, cost-effectiveness and potential for *in-situ* testing with the developed biosensor provide a valuable tool for the detection of OsHV-1 in aquaculture facilities, improving their management.

Keywords (6): Ostreid herpesvirus 1 (OsHV-1); isothermal recombinase polymerase amplification (RPA); electrochemical biosensor; Pacific oyster; *Crassostrea gigas*.

Highlights

- The first electrochemical biosensor to detect OsHV-1 DNA in *Crasosstrea gigas* is described.
- The strategy is based on isothermal recombinase polymerase amplification.
- The system achieves a limit of detection of 207 OsHV-1 target copies.
- A good correlation with qPCR in the analysis of OsHV-1 DNA in 16 oyster samples is achieved.

Graphical abstract



1. Introduction

Shellfish aquaculture has become an important component of the aquaculture industry, representing 40% of the aquaculture production in terms of weight and 28% in value in Europe. However, the shellfish industry has suffered from slow growth as a consequence of various infectious diseases [1]. Among pathogens affecting molluscs, ostreid herpesvirus 1 (OsHV-1) [2] is currently one of the major threats to shellfish aquaculture, particularly to the production of Pacific oysters (*Crassostrea gigas*) [3-5]. Recurrent mass mortality outbreaks of *C. gigas* have been reported worldwide, especially in European counties such as France [6] and Spain [7], but also in Australia [8] and New Zealand [9], where industry is suffering from this disease. Since oysters are farmed in open waters and there are neither vaccination nor antiviral therapies available, the efforts of the sector to manage the impact of this virus are essentially focused on the development of prevention strategies, mitigation approaches and early diagnostic tools.

Traditionally, surveillance of OsHV-1 has relied on the detection of the virus in oysters through conventional molecular techniques such as PCR [10] and *in-situ* hybridisation [11]. However, these techniques are time-consuming and laborious, and also suffer from low sensitivity and specificity. Recently, quantitative PCR (qPCR) protocols have been developed for OsHV-1 detection [12], and are routinely used for OsHV-1 screening. Despite being sensitive and specific, qPCR requires a highly precise heat source to perform the thermal cycling as well as an expensive fluorescent detector, limiting its practical use for deployment for use in the field. Therefore, the development of rapid, user-friendly and cost-effective analysis tools is of great importance to better anticipate the presence of OsHV-1 and ultimately mitigate the effects of the disease.

Isothermal DNA amplification techniques have emerged in recent years as an effective replacement of PCR technology for molecular diagnostics [13]. These techniques achieve DNA amplification at a constant temperature, facilitating their combination with miniaturised analytical devices to perform *in-situ* tests. To date, several isothermal methods exist including nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), helicase dependent amplification (HDA) and recombinase polymerase amplification (RPA), which have been successfully applied in several areas including medicine, agriculture, aquaculture and food safety. Specifically for OsHV-1 detection, only LAMP followed by gel electrophoresis/fluorescent read-out [14] and RPA with real-time fluorescent detection [15] have been reported. Although both techniques allow sensitive and specific detection within a few minutes (20-40 min), LAMP is performed at a relatively high temperature (60-65 °C) and requires the design of 4 primers, whereas RPA can be conducted at a much lower temperature (37-40 °C) and only requires 2 primers, rendering it particularly attractive. However, the recently described RPA methodology for OsHV-1 detection, while effective, still requires the use of expensive and bulky optical detection equipment, thereby efforts

should be devoted to the development of cost-effective and miniaturised systems for OsHV-1 detection that can be easily deployed in the field. Electrochemical biosensors can certainly address this demand since they do not require complex instrumentation, handle small volumes of sample and can be integrated into microfluidic platforms for subsequent automation [16]. Although there are few reports describing the combination of RPA with electrochemical detection [17-20], this promising strategy is still in its infancy, and efforts need to be focused on developing new proof-of-concept strategies and demonstrating their applicability in real-life settings.

Within this context, we propose a simple, low-cost and miniaturised biosensor that combines RPA with an electrochemical read-out for the detection of OsHV-1 (Fig. 1). Our strategy starts with isothermal DNA amplification using primers modified with tails. A carbon stopper is located between the tail and the primer in order to prevent further elongation of the tail during amplification. Hence, amplification results in a double-stranded DNA (dsDNA) product with single-stranded DNA (ssDNA) tails at each end (Fig. 1a). This configuration allows subsequent detection of the RPA product through a capture probe immobilised on an electrode *via* a thiol group (which hybridises to one tail) and a reporter probe labelled with horseradish peroxidase (HRP) (which hybridises to the other tail) (Fig. 1b). A colorimetric approach was first used to optimise the RPA conditions. The system was then transferred to a thin-film gold electrode for development of the corresponding DNA-based amperometric biosensor. Finally, the biosensor was applied to the analysis of OsHV-1 DNA in Pacific oyster (*C. gigas*) samples, and quantifications were compared with qPCR analysis.

2. Materials and methods

2.1. Reagents and solutions

Custom oligonucleotide primers and probes were synthesised by Biomers (Ulm, Germany). TwistAmp Basic kit was obtained from TwistDx Ltd. (Cambridge, UK). Qiagen DNeasy Blood and Tissue kit, QIAquick gel extraction kit, GeneJET PCR purification kit, Taq DNA polymerase, SYBR green and pierce maleimide-activated microtitre plates were supplied by Thermo Fisher Scientific (Madrid, Spain). Tween-20, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, 6-mercapto-1-hexanol, skimmed milk powder, potassium phosphate dibasic, potassium phosphate monobasic, sodium chloride, sulphuric acid, hydrogen peroxide and all other reagents were acquired from Sigma-Aldrich (Tres Cantos, Spain).

2.2. Equipment

Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. (Vermont, USA). Gene 5 was used to collect and evaluate data.

Thin-film gold single electrodes (ED-SE1-Au) and a Drop-cell connector were provided by MicruX Technologies (Gijón, Spain). Electrodes (10 x 6 x 0.75 mm) are fabricated on a glass substrate and consist of a gold working electrode of 1 mm in diameter with its own gold counter electrode and gold reference electrode and a resin protective layer is used to delimit the electrochemical cell. Chronoamperometric measurements were performed with an AUTOLAB PGSTAT128N potentiostat from Metrohm Autolab (Utrecht, The Netherlands). Data were collected and evaluated with Nova 2.1.4 software.

PCR and RPA reactions were performed using a Nexus Gradient Thermal Cycler from Eppendorf Ibérica (Madrid, Spain). qPCR reactions were performed in 7300 real-time PCR system (Thermo Fisher Scientific, Madrid, Spain) and 7300 system 1.4.0 software was used to collect and evaluate data. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) was used to quantitatively and qualitatively check amplified and extracted DNA.

2.3. Target OsHV-1 DNA

Target OsHV-1 DNA was prepared from OsHV-1 virus particles isolated using anionic magnetic beads [21]. Genomic OsHV-1 DNA from the captured particles was extracted with the Qiagen DNeasy Blood and Tissue kit as reported in our previous work [21]. Afterwards, target DNA was amplified from genomic OsHV-1 DNA using the ORF95F/ORF95R primer set described by Gao and collaborators (Table S1). Each 25-µL reaction mixture contained 2.5 µL PCR Buffer 1X, 600 µM dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1 U of Taq polymerase

and 2 µL of genomic OsHV-1 DNA. PCR conditions included 40 cycles of 95 °C for 30 s followed by primer annealing at 65 °C for 30 s and extension at 72 °C for 1 min. PCR products were checked by agarose gel electrophoresis, purified using the QIAquick gel extraction kit and quantified using a NanoDrop to calculate the target OsHV-1 DNA concentration. DNA was stored at -20 ˚C until use.

2.4. Pacific oyster samples

Spat *C. gigas* oysters (*n* = 16) were obtained from an infectivity experiment described in our previous work [21]. Oyster samples were sourced from two aquaria: a control aquarium (oysters injected with sterile water) and a treatment aquarium (oysters injected with naturally OsHV-1-contaminated oyster homogenate). Mortality was checked daily, and any dead/moribund oysters being removed from the treatment aquarium (*n* = 6). At day 11, alive oysters were also collected from both control (*n* = 5) and treatment (*n* = 5) aquaria. Total genomic DNA from 16 oyster samples was extracted using the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's protocols with 100 µL of AE buffer used for the final elution step [21]. DNA quality and quantity were measured using a NanoDrop. DNA was diluted to 50 ng of total OsHV-1 DNA/µL (to be analysed by qPCR) or to 100 ng of total OsHV-1 DNA/µL (to be analysed by the biosensor) and stored at -20 °C until use.

2.5. Primers and probes

The primers used in this work were based on the ORF95F/ORF95R OsHV-1 real-time RPA primer set reported by Gao and collaborators [15]. Primers have a length of 30 bp and amplify a product of 164 bp (Table S1). The specificity of the primers for OsHV-1 was confirmed, showing any interferences from a variety of viruses and bacteria that commonly infect bivalve molluscs [15]. In this study, these primers were modified with oligonucleotide tails, which enable the direct detection of the RPA product through complementary capture and reporter probes (Fig. 1). Primer and probe sequences are detailed in Table 1. Tails and probes were tested to confirm the absence of non-specific binding with the primer sequences and the OsHV-1 genome (AY509253.2) [2] using Multiple Primer analyser software.

2.6. Recombinase polymerase amplification (RPA)

The RPA of OsHV-1 DNA was performed using the TwistAmp Basic kit. Following systematic optimisation, the RPA reaction (50 µL) contained 14.75 µL of rehydration buffer, ½ enzyme pellet, 120 mM of each primer, 14 mM of magnesium acetate and 5 µL of DNA, which corresponded to: a) target OsHV-1 DNA for the RPA optimisation and the storage stability of the capture probe-modified electrodes (10^5 OsHV-1 copies) and for the construction of the calibration curves (10-fold dilutions, from 10^8 to 10^2 OsHV-1 copies); and b) DNA extracted from oyster samples (100 ng of total OsHV-1 DNA/µL). Magnesium acetate was added to initiate the

RPA reaction. Reactions were performed at 37 ˚C for 30 min. Blanks (NTC = no template control) were included in all experiments.

Optimisation of the RPA conditions was carried out using colorimetric detection (section 2.7). Primer concentration (2-fold dilutions, from 480 to 120 mM) and the need to clean-up the RPA product before detection were evaluated, while maintaining all other RPA parameters constant. Purification of the RPA product was carried out using the GeneJET PCR purification kit according to the manufacturer's instructions, with 50 µL of TE buffer for the final elution step. Once the optimal conditions were selected, the effect of RPA reaction time (30, 40 and 60 min) on the LOD was investigated.

2.7. Colorimetric assay

Colorimetric assays were carried out on 96-well maleimide-activated microtitre plates. Microtitre wells were incubated with 50 µL of 500 nM thiolated capture probe in PBS (100 mM phosphate, 150 mM NaCl, pH 7.4). Blocking of any non-functionalised maleimide groups was achieved using 200 µL of 100 µM 6-mercapto-1-hexanol in Milli-Q water. A subsequent blocking step was performed by the addition of 200 µL of 5% w/v skimmed milk in PBS. In the following step, 45 µL of RPA product were added and, subsequently, 50 µL of 10 nM HRP-conjugated reporter probe in PBS-Tween (100 mM potassium phosphate, 150 mM NaCl, 0.05% v/v Tween-20, pH 7.4) were incubated. Finally, 100 µL of TMB liquid substrate were added, and 10 min later the absorbance was read at 620 nm. After each step, wells were rinsed three times with 200 µL of PBS-Tween. During incubations, microtitre plates were placed on a plate shaker. All steps were carried out at room temperature for 30 min except for the immobilisation of the capture probe, which was performed at 4 °C overnight.

2.8. Electrochemical biosensor

The electrochemical biosensor protocol was essentially the same as the colorimetric assay with the exception of some minor volume adjustments for use with the MicruX electrodes in the detection step. Regarding volumes, 10 µL of non-purified RPA product were placed on each working electrode, whereas 50 µL were used in all other steps. For the electrochemical measurement, 2 µL of TMB were added to each electrode and incubated for 5 min and, finally, the TMB reduction current was measured using chronoamperometry applying -0.2 V for 5 s. After each step, electrodes were rinsed, first with PBS-Tween and then with Milli-Q water, and finally dried. All steps were carried out at room temperature for 30 min except for the immobilisation of the capture probe, which was performed at 4 °C overnight in a water-saturated atmosphere.

Following electrochemical measurements, electrodes were cleaned by immersion in Piranha solution (3:1 H_2SO_4:H_2O_2) rinsed with Milli-Q water, dried and stored at room temperature for subsequent use. To evaluate the stability of the functionalised electrodes, gold electrodes modified with the thiolated capture probe were prepared as described before, washed and stored at 4 °C and −20 °C. The initial electrochemical response (reference value) and the response after 7 and 17 days was measured.

2.9. Quantitative PCR (qPCR) analysis

Quantification of OsHV-1 DNA in oyster samples by qPCR was performed as described in our previous work [21]. Briefly, each 20-µL reaction mixture contained 10 µL 2X SYBR Green dye, 0.5 µM OsHVDPFor/OsHVDPRev primers [10] (Table 1. SI) and 1 µL of extracted DNA from oyster samples (50 ng of total OsHV-1 DNA/µL). The qPCR conditions included 45 cycles of amplification following a three-step protocol (95 °C for 30 s, 60 °C for 1 min and 72 °C for 45 s) and a final step for melting temperature curve analysis at 60 °C for 1 min with a gradual increase of temperature (1 °C/15 s). Quantification of OsHV-1 DNA copies was carried out using an interlaboratory-validated standard curve based on 10-fold dilutions of OsHV-1 plasmid DNA.

2.10. Data analysis

Measurements were performed in triplicate for the colorimetric assay and qPCR and in quadruplicate for the electrochemical biosensor. Calibration curves obtained using the colorimetric assay and the electrochemical biosensor were fitted to a sigmoidal logistic four-parameter equation using SigmaPlot 12.0 software. The LOD was defined as the blank (NTC) signal plus 3-fold its standard deviation. OsHV-1 DNA quantifications in oyster samples obtained by the biosensor and qPCR are expressed as OsHV-1 DNA copies per 50 ng of total DNA. Linear regression was used to evaluate the correlation between OsHV-1 DNA quantifications determined with the electrochemical biosensor and those obtained from qPCR analysis. Correlation between both techniques was assessed by means of Pearson's correlation coefficient (*r*). Data analyses were performed with SigmaStat 3.1 software.

3. Results and discussion

3.1. Colorimetric assay and RPA optimisation

Due to the similar affinity of maleimide and gold for thiol groups, colorimetric assays on maleimide-coated microtitre plates were first developed to evaluate the feasibility of the approach and to optimise the experimental parameters of the RPA.

According to the TwistDx manual, purification of RPA products before their detection is required to avoid non-specific signals. However, DNA purification involves an extra step, adding complexity, reagents and an equipment requirement to the overall process. With the aim of simplifying the protocol, both the need to purify RPA products and the effect of the RPA primer concentration were evaluated using the colorimetric assay, with a positive control consisting of a non-saturated concentration of OsHV-1 copies (i.e. 10^5 OsHV-1 copies) and a negative control (NTC).

The effect of using non-purified RPA product was first investigated (Fig. 2). When the primer concentration recommended by the manufacturer (480 mM) was used, the absorbance value corresponding to the non-specific adsorption (NTC) was as high as that achieved with the positive control. By reducing the primer concentration by half (240 mM), the non-specific adsorption value was also reduced by half, while the positive signal was maintained. Finally, the use of 120 mM primer concentration resulted in a greatly reduced non-specific adsorption (non-detectable signal), whereas the positive signal was maintained again. These results suggest that the presence of residual primers may cause the formation of primer-dimers, leading to an increase of the non-specific adsorption values in our configuration. When RPA products were purified, a decrease in the specific signals was observed for all primer concentrations tested when compared to the responses obtained with non-purified ones, suggesting that the purification step may cause some loss of RPA product. On the other hand, although non-specific adsorption values were always lower than their corresponding positive ones, they were still significant at the highest primer concentration. This could indicate that not all residual primers are efficiently removed when purifying. In summary, the use of non-purified RPA product in combination with the lowest primer concentration tested provided the highest NTC-subtracted signals, thereby these conditions were chosen for the construction of the calibration curve.

The calibration curve constructed using target OsHV-1 DNA (from 10^8 to 10^1 OsHV-1 target copies) is shown in Figure 3. An LOD of 426 OsHV-1 target copies was achieved, which was higher than other reported molecular methods for OsHV-1 detection (see section 3.2). With the aim of decreasing the LOD the system, the effect of extending the RPA reaction time to 40 and 60 min was evaluated. However, the same LODs were reached,

thus the reaction time was maintained to 30 min, as recommended by the manufacturer and also used in our previous works [22, 23].

The use of an isothermal amplification method allows reducing the amplification time and the power needed, making the system more compatible for integration into miniaturised devices. Additionally, the optimisation of primer concentration in RPA enables the avoidance of a purification step of amplified products prior to their detection, thus reducing costs, time and complexity to the assay.

3.2. Electrochemical biosensor

After protocol optimisation using the colorimetric assay, the strategy was transferred to gold electrodes to develop the corresponding electrochemical DNA-based biosensor. Maleimide plates were replaced by thin-film gold electrodes and the electrochemical signal was recorded instead of the colorimetric one. The use of miniaturised electrodes enabled a 1/5 reduction of the volume of RPA product required.

Fitting the calibration curve to the sigmoidal logistic four-parameter equation (Fig. 4) resulted in an LOD of 207 OsHV-1 target copies, which is ~2-fold lower than that obtained with the colorimetric approach. The biosensor has an LOD lower than that attained with PCR followed by gel electrophoresis (1000 copies) [10], but higher than those achieved by other molecular methods described to date, including qPCR (4 copies) [12], LAMP (20 copies) [14] or real-time RPA (5 copies) [15]. Nevertheless, the biosensor offers potential advantages in terms of portability, miniaturisation and automation, which make it more applicable to field detection.

Repeatability (intra-day precision) was appropriate, with relative standard deviation (RSD) values of 7.4 and 3.8% at 105 and 108 OsHV-1 target copies, respectively. Reproducibility (inter-day precision) was also appropriate, with RSD values of 21.6 and 6.8% at the same copies. Higher RSD values were obtained at intermediate number of copies due to the high sensitivity of the biosensor. The use of signal amplification strategies or pre-concentrating agents such as MBs can be explored to decrease the LOD of the electrochemical biosensor as well as to improve the RSD values.

3.3. Stability of the functionalised electrodes

To investigate the possibility to shorten the biosensor protocol time, the storage stability of the capture probe-modified electrodes was tested at 4 and -20 °C over 17 days. The intensity values achieved were constant with time (Fig. S1), clearly demonstrating that the functionalised electrodes were stable for at least 17 days, stored both at 4 °C and −20 °C. Moreover, such real-time stability can be used to predict shelf-life of functionalised electrodes using the Q Rule method, which states that a product degradation rate changes exponentially with the temperature, and is proportional to $(Q10)^n$, where n is the temperature change divided by 10 [24]. The

value of Q10 is typically set at 2, 3, or 4, which correspond to reasonable activation energies. In this work, taking into account that the functionalized electrodes are stable at least for 17 days, an *n* value of 2.4 and a conservative Q10 value of 2, the predicted stability [17 days/$(2)^{2.4}$] of the product at -20 °C is at least 3 months. This great storage stability significantly reduces the assay time, as multiple electrodes can be prepared on the same day and stored until use, providing ready-to-use modified electrodes and allowing to perform the assay within the same day.

3.4. OsHV-1 detection in Pacific oyster samples

A total of 16 oyster samples from an experimental infection were analysed using the electrochemical biosensor to demonstrate its applicability. OsHV-1 target copies in the oysters were quantified using the sigmoidal logistic equation. As shown in Table 2, samples from the control aquarium were all negative, and 2 out of 11 samples from the treatment aquarium also provided negative results. Such negative oysters were alive when collected at the end of the experiment. The rest of the oysters from the treatment aquarium, which were dead/moribund or alive, resulted positive for OsHV-1 detection, with contamination levels from 1.50 x 10^2 to 3.34 x 10^5 OsHV-1 DNA copies/50 ng of total DNA. As expected, dead/moribund oysters presented higher viral DNA amounts than the live ones.

Quantifications obtained using the biosensor were compared with those obtained by qPCR analysis. qPCR also provided negative results for the control oysters, whilst for the oysters from the treatment aquarium, OsHV-1 DNA was detected in all samples. The two samples that resulted negative using the biosensor were positive by qPCR, certainly due to the higher LOD of the biosensor. Linear regression was used to evaluate the correlation between techniques. Samples that resulted negative for both techniques were not included in the regression (n = 5), neither samples that were negative for one of the two techniques (n = 2). Samples with ~10^4 OsHV-1 DNA copies/50 ng of total DNA showed an excellent 1:1 correlation, while a slight overestimation and underestimation by the biosensor was observed in samples with <10^3 and >10^5 DNA copies/50 ng of total DNA, respectively. Nevertheless, as shown in Figure 5, the overall correlation between techniques was highly significant (Pearson's r = 0.988; P < 0.001).

Our innovative biosensor configuration offers several advantages. Firstly, the use of an isothermal DNA amplification such as RPA circumvents the need for equipment-dependent and time-consuming PCR technology. Secondly, the electrochemical read-out instrumentation is less complex than the currently used optical detection systems. Thirdly, the use of miniaturised thin-film electrodes enables working with smaller sample volumes. Although not yet automated, the system offers great potential to be easily integrated into microfluidic systems to develop compact devices that could be used in the field by end-users. The economic loses associated to the OsHV-1 infection in aquaculture facilities make this bioanalytical tool highly interesting

for screening and quantification purposes. Examples of applicability could be the fast screening of stocks before they are transferred to a depuration plant or another harvesting area, or the "on the spot" control by competent customs agencies.

4. Conclusions

In this work, an electrochemical biosensor for the detection of OsHV-1 has been described. Prior to biosensor development, RPA conditions were optimised using a colorimetric assay. The biosensor exhibits a good analytical performance, with the specificity, storage stability, sensitivity and reliability necessary to be applied to the detection of the pathogen in oyster samples. This was evidenced by the strong correlation found between the OsHV-1 DNA concentrations determined by the biosensor and qPCR in the analysis of oyster samples.

The present work not only reports the first electrochemical biosensor for OsHV-1 detection, but also constitutes a clear demonstration that progress in molecular biology in conjunction with simple detection systems can result in powerful analytical tools with wide applications. Future work will be focused on decreasing LODs by means of improving both the amplification and the detection steps or, alternatively, using pre-concentrating agents such as magnetic beads.

Acknowledgments

This work was supported by the European Commission H2020 Framework Programme through grant agreement 678589 (VIVALDI project). The authors are grateful to Dr. K. B. Andree for his initial suggestions. The authors also acknowledge support from the CERCA Programme/Generalitat de Catalunya. Anna Toldrà acknowledges IRTA-Universitat Rovira i Virgili-Banco Santander for her PhD grant (2015PMF-PIPF-67).

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Table 1. List of primers (underlined) and probes and their respective modifications.

Name	**Sequence (5'-3')**
OsHV-1 forward primer with tail	gtt ttc cca gtc acg ac-C3-cat gtt tac gtg gaa atg ttg gat tgg cta
OsHV-1 reverse primer with tail	tgt aaa acg acg gcc agt-C3-atg tca aat agg ttg ttg gca gtg atg gtc
Capture probe	gtc gtg act ggg aaa act ttt ttt ttt ttt tt-C3-SH
Reporter probe	HRP-act ggc cgt cgt ttt aca

Table 2. OsHV-1 DNA concentrations (OsHV-1 DNA copies/50 ng of total DNA) in oysters obtained by the electrochemical biosensor and qPCR analysis.

Sample	Oyster physical state	Aquarium	Electrochemical biosensor	qPCR
1	dead/moribund	treatment	$3.34 \times 10^5 \pm 5.91 \times 10^4$	$7.13 \times 10^5 \pm 4.05 \times 10^4$
2	dead/moribund	treatment	$4.78 \times 10^5 \pm 1.02 \times 10^5$	$4.81 \times 10^5 \pm 7.65 \times 10^4$
3	dead/moribund	treatment	$7.26 \times 10^4 \pm 1.52 \times 10^4$	$1.79 \times 10^5 \pm 4.10 \times 10^4$
4	dead/moribund	treatment	$6.10 \times 10^3 \pm 4.76 \times 10^2$	$6.52 \times 10^3 \pm 2.34 \times 10^3$
5	dead/moribund	treatment	$5.21 \times 10^3 \pm 6.80 \times 10^1$	$4.38 \times 10^3 \pm 1.37 \times 10^3$
6	dead/moribund	treatment	$2.75 \times 10^3 \pm 6.18 \times 10^2$	$1.98 \times 10^3 \pm 4.46 \times 10^2$
7	alive	treatment	$1.97 \times 10^2 \pm 6.89 \times 10^1$	$1.21 \times 10^2 \pm 2.46 \times 10^1$
8	alive	treatment	$5.27 \times 10^2 \pm 6.98 \times 10^1$	$7.83 \times 10^1 \pm 1.97 \times 10^1$
9	alive	treatment	$1.50 \times 10^2 \pm 4.47 \times 10^1$	$3.24 \times 10^1 \pm 5.67$
10	alive	treatment	nd	$1.04 \times 10^1 \pm 5.83$
11	alive	treatment	nd	$7.93 \times 10^1 \pm 3.38$
12	alive	control	nd	nd
13	alive	control	nd	nd
14	alive	control	nd	nd
15	alive	control	nd	nd
16	alive	control	nd	nd

nd: not detected

Figure 1. Schematic representation of the strategy: **a**) recombinase polymerase amplification and **b**) electrochemical biosensor.

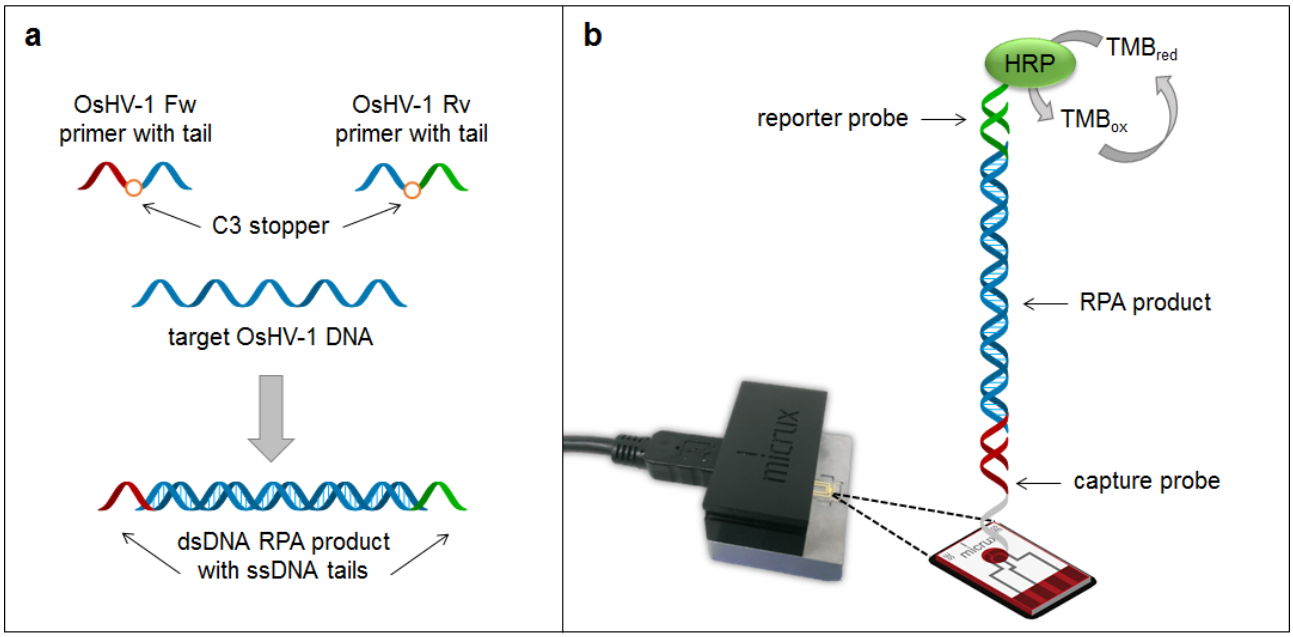


Figure 2. Optimisation of the RPA conditions by the colorimetric assay using 10^5 OsHV-1 target copies and NTC. Black bars = purified RPA products; grey bars = non-purified RPA products. Error bars are the standard deviation of the mean, *n* = 3.

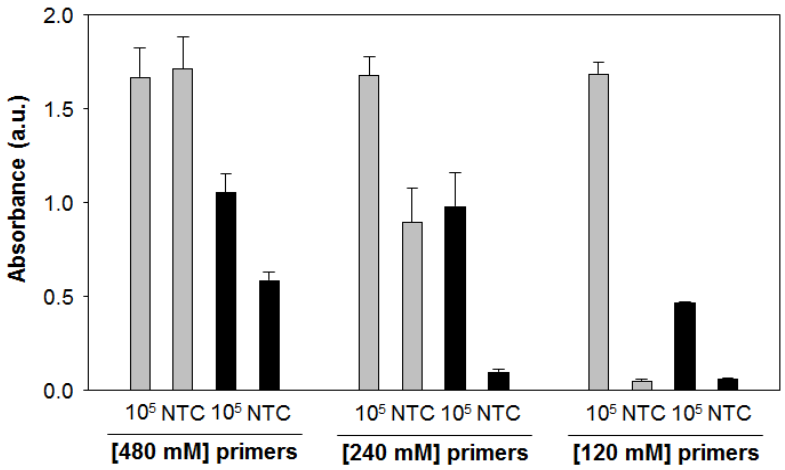


Figure 3. Calibration curve obtained with the colorimetric assay. Errors bars are the standard deviation of the mean, *n* = 3.

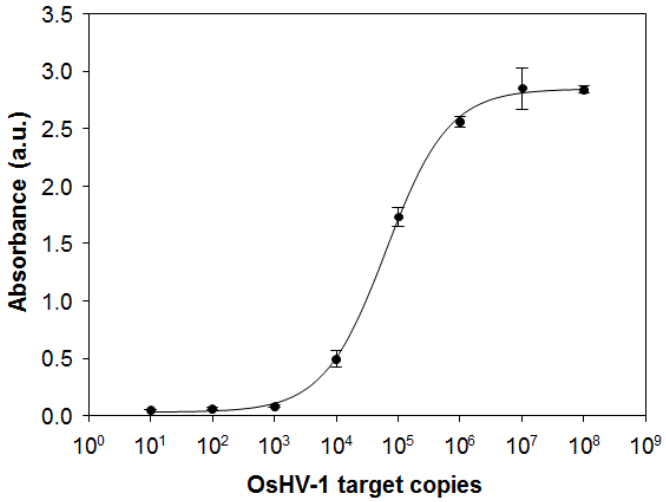


Figure 4. Calibration curve obtained with the electrochemical biosensor. Error bars are the standard deviation of the mean, *n* = 4.

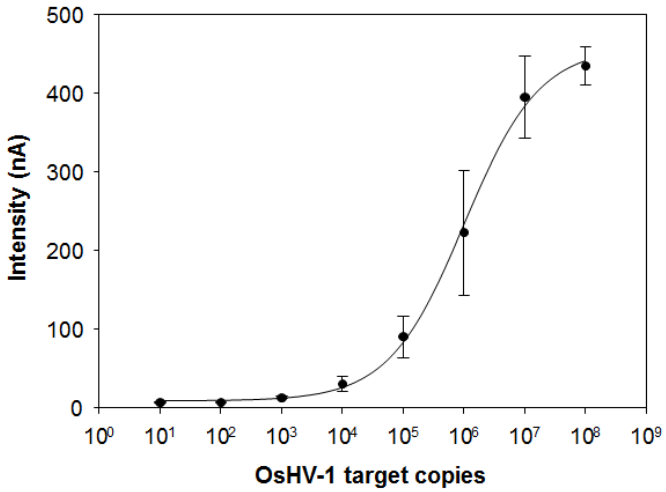
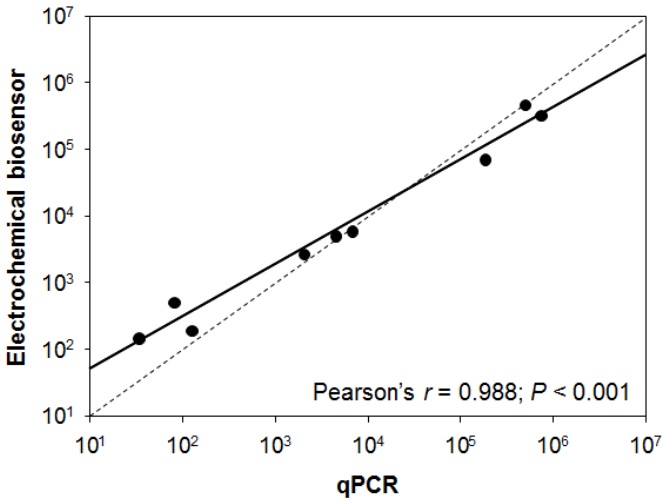


Figure 5. Correlation between OsHV-1 DNA concentrations (OsHV-1 DNA copies/50 ng of total DNA) in oysters obtained with the electrochemical biosensor and q PCR analysis (*n* = 9) (black line). Oyster samples that were negative for both techniques were not included in the regression (*n* = 5), neither samples that were negative for one of the two techniques (*n* = 2). The dashed line represents the bisector, to which points should align in case of perfect identity of the methods.



APPENDIX. SUPPLEMENTARY MATERIAL

Table S1. List of primers used for PCR (section 2.3) and qPCR (section 2.9) reactions.

	Primer name	Sequence (5'-3')	Primer size (bp)	Amplicon size (bp)	Target gene
PCR OsHV-1 forward primer	ORF95F[1]	cat gtt tac gtg gaa atg ttg gat tgg cta	30	164	ORF95
PCR OsHV-1 reverse primer	ORF95R[1]	atg tca aat agg ttg ttg gca gtg atg gtc	30		
qPCR OsHV-1 forward primer	OsHVDPFor[2]	att gat gat gtg gat aat ctg tg	23	197	ORF100
qPCR OsHV-1 reverse primer	OsHVDPRev[2]	ggt aaa tac cat tgg tct tgt tcc	24		

[1] From: F. Gao, J.Z. Jiang, J.Y. Wang, H.Y. Wei, Real-time quantitative isothermal detection of Ostreid herpesvirus-1 DNA in *Scapharca subcrenata* using recombinase polymerase amplification, J. Virol. Methods 255 (2018) 71-75.

[2] From: S.C. Webb, A. Fidler, T. Renault, Primers for PCR-based detection of ostreid herpes virus-1 (OsHV-1): application in a survey of New Zealand molluscs, Aquaculture 272(1-4) (2007) 126-139.

Figure S1. Storage stability of the capture probe-modified gold electrodes. Bars represent the electrochemical responses obtained with electrodes stored at 4 °C (black bars) and at -20 °C (grey bars) respect to day 0 (white bar, reference value). Error bars are the standard deviation of the mean, *n* = 4.