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Propidium monoazide PCR, a method to determine OsHV-1 undamaged capsids and to estimate virus Lethal Dose 50*

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ABSTRACT

Ostreid herpes virus 1 (OsHV-1) has been classified within the *Malacoherpesviridae* family from the *Herpesvirales* order. OsHV-1 is the etiological agent of a contagious viral disease of Pacific oysters, *C. gigas*, affecting also other bivalve species. Mortality rates reported associated with the viral infection vary considerably between sites and countries and depend on the age of affected stocks. A variant called µVar has been reported since 2008 in Europe and other variants in Australia and in New Zealand last decade. These variants are considered as the main causative agents of mass mortality events affecting *C. gigas*.

Presently there is no established cell line that allows for the detection of infectious OsHV-1. In this context, a technique of propidium monoazide (PMA) PCR was developed in order to quantify "undamaged" capsids. This methodology is of interest to explore the virus infectivity. Being able to quantify viral particles getting an undamaged capsid (not only an amount of viral DNA) in tissue homogenates prepared from infected oysters or in seawater samples can assist in the definition of a Lethal Dose (LD) 50 and gain information in the experiments conducted to reproduce the viral infection.

The main objectives of the present study were (i) the development/optimization of a PMA PCR technique for OsHV-1 detection using the best quantity of PMA and verifying its effectiveness through heat treatment, (ii) the definition of the percentage of undamaged capsids in four different tissue homogenates prepared from infected Pacific oysters and (iii) the approach of a LD50 during experimental viral infection assays on the basis of a number of undamaged capsids. Although the developped PMA PCR technique was unable to determine OsHV-1 infectivity in viral supensions, it could greatly improve interpretation of virus positive results obtained by qPCR. This technique is not intended to replace the quantification of viral DNA by qPCR, but it does make it possible to give a form of biological meaning to the detection of this DNA.

1. Introduction

Herpesviruses infecting marine mollusks have attracted particular research attention due to their economic and ecological impact during the past 30 years. A particular herpesvirus has been purified from naturally infected larval Pacific oysters collected in 1995 in a French commercial hatchery during a mass mortality outbreak (Le Deuff and Renault, 1999) and its genome entirely sequenced (Davison et al., 2005). This virus has been classified as ostreid herpesvirus type 1 (OsHV-1) within the *Malacoherpesviridae* family from the *Herpesvirales* order (Davison et al., 2009). OsHV-1 is the etiological agent of a contagious viral disease of Pacific oysters, *Crassostrea gigas*, affecting also other bivalve species. Mortality rates reported associated with the viral infection vary between sites and countries and depend on the age of affected stocks. A variant called µVar was reported since 2008 in Europe (Domeneghetti et al., 2014; Lynch et al., 2012; Martenot et al., 2011; Peeler et al., 2012; Renault et al., 2012; Roque et al., 2012; Segarra et al., 2010) and other variants in Australia (Jenkins et al., 2013; Paul-Pont et al., 2013, 2014; Trancart et al., 2022) and in New Zealand (Keeling et al., 2014; Renault et al., 2012) last decade. These variants are considered as the main causative agents of mass mortality events affecting *C. gigas*.

OsHV-1 like vertebrate herpesviruses is composed of a relatively large (207 kbp), double-stranded, linear DNA genome (Davison et al.,

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2005) encased within an icosahedral capsid, which is itself wrapped in a tegument and an envelope (Renault et al., 2001a and b). The research into virus cytopathogenic effects in cell cultures is not practical due to the absence of marine mollusk cell lines. Moreover, all assays carried out to cultivate OsHV-1 in fish, insect and mammal cell lines as well as in *Biomphalaria glabrata* embryo (Bge) cells failed (Renault, 2011). In this context, although Pacific oyster primary cell cultures or *ex vivo* cells could be used to conduct experiments (Delisle et al., 2023; Morga et al., 2017), producing infective virus stocks *in vitro* is not possible. Exploring viral infectiosity remains a time-consuming work passing through experimental infections in laboratory conditions (Schikorski et al., 2011a, b). Experimental infections are based on the use of oyster tissue homogenates as a virus source. OsHV-1 DNA is quantified by real time PCR in these tissue homogenates before carrying out the assays. However, viral DNA quantification does not give exact information about infectious particles present as viral DNA can be contained in damaged particles or can be free outside of capisds.

A technique of PMA PCR was developed in the laboratory in order to quantify "undamaged" (not permeable to PMA) OsHV-1 capsids in presence of a mixture of pesticides (Moreau et al., 2015). While the PMA PCR technique does not allow exploring viral envelop integrity, it should allow exploring capsid integrity. For herpesviruses, a particle is infectious if it is intact, i. e. it gets undamaged envelop and capsid. Being able to quantify viral particles possessing an undamaged capsid (not only an amount of viral DNA) in oyster tissue homogenates or in seawater samples can assist in the definition of a Lethal Dose 50 (LD50). Indeed, assays conducted today are based on quantification of viral DNA. While it is clear that the present PMA PCR technique does not allow to define a number of viral particles with an intact envelope, it does make it possible to refine the knowledge of virus infectivity by not taking into account free viral DNA or DNA contained in damaged capsids.

In this context, the objectives of this study were (i) first to develop/optimize a PMA PCR technique for OsHV-1 using the best quantity of PMA and verifying its effectiveness through heat treatment of samples, (ii) to define the percentage of undamaged capsids in four different tissue homogenates of infected oysters, and (iii) to estimate a LD50 during experimental viral infections on the basis of a number of undamaged capsids and not on a number of viral genome copies contained in the viral source (tissue homogenate).

Thus through a method to determine OsHV-1 undamaged capsids and to estimate virus LD50, we implemented a method to optimized the detection of infectious OsHV-1. In the context of the long-lasting question regarding the interpretation of a genomic detection by PCR, this method can be used to better understand the meaning of the detection of viral DNA in a sample, in particular as a function of the stage of infection.

2. Material and Methods

2.1. PMA and viral suspension preparation

The stock solution was prepared from 1 mg of PMA^{TM} (propidium monoazide) (Biotium, Hayward, CA) solubilized in 100 µL of a solution of 20 % dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co) at a concentration of 20 mM. The three final PMA concentrations selected were 300 µM, 400 µM and 500 µM.

Four tissue homogenates containing OsHV-1 (A–D) were prepared according to the protocol described by Schikorski et al. (2011a, b). In brief, initial tissue homogenates were prepared using ten infected Pacific oysters. To verify that oysters were OsHV-1 infected, a small piece of mantle was sampled from each individual and used for OsHV-1 detection by real time PCR. Gills and mantle were dissected and pooled together in a 50 mL sterile tube. All subsequent dilutions were made using 0.22 µm filtered artificial seawater (ASW). Tissues were crushed on ice using an Ultraturax mixer (3 × 5 s). After centrifugation (1000 g, 5 min, 4 °C), supernatants were placed in a new tube and diluted by addition of 4 volumes of ASW. Finally, the clarified tissue homogenates were filtered consecutively in sterile conditions using syringe filters at 5 µm, 2 µm, 0.45 µm and 0.22 µm pore sizes (Millipore, Billerica, USA). Viral suspension dilutions were then prepared in sterile artificial seawater as previously reported (Schikorski et al., 2011a, b). OsHV-1 suspensions were thus used undiluted and diluted to 1/10, 1/100, 1/1 000 and 1/10 000.

2.2. PMA treatment and viral DNA extraction

Two hundred and eighty µL of each virus suspension (undiluted and dilutions) were tested. A volume of PMA stock solution to achieve the desired final concentrations and sterile artificial seawater were added in order to get a reaction volume of 300 µL. Samples without PMA consisted of 280 µL and 20 µL of sterile seawater and viral suspension, respectively and served as controls. Samples were placed 10 min under stirring in the dark and then exposed to the lamp of the photo-activation system PhAST Blue (GenIUL) for 10 min. Additionally, samples of viral suspensions containing PMA without photoactivation were used to confirm the absence of PMA effect on PCR amplification. In the presence of PMA, the lysis phase for DNA extraction from OsHV-1 suspensions was carried out in the dark. DNA extraction was performed with QIAamp DNA miniKit (QIAgen) according to the manufacturer's protocol.

2.3. Quantification of OsHV-1 DNA

Real time quantitative PCR was performed in duplicate using a Mx3000 Thermocycler (Agilent). Amplification reactions were performed in a total volume of 20 µL. Each well contained 5 µL of extracted DNA, 10 µL of Brilliant III Ultra-Fast SYBR®Green PCR Master Mix (Agilent), 2 µL of each primer (OsHVDPFor 5'-ATTGATGATGTGGATAATCTGTG-3' and OsHVDPRev 5'-GGTAAATACCATTGGTCTTGTTCC-3') (Webb et al., 2007) at the final concentration of 550 nM each, and 1 µL of distilled water. Real time PCR cycling conditions were as follow: 3 min at 95 °C, followed by 40 cycles of amplification at 95 °C for 5 s, 60 °C for 20 s. The results were expressed as a Ct value (Cycle threshold) or as a log10 of OsHV-1 copies per µL of viral suspension. Absolute quantification of OsHV-1 DNA copies was carried out by comparing CT values obtained for tested samples with the standard curve based on a ten-fold dilution curve derived from a stock solution of OsHV-1 genomic DNA (5.10^6 copies/µL) extracted from purified virus particles (Le Deuff and Renault, 1999).

2.4. Effect of PMA concentration

The effect of different PMA concentrations on the amplification of viral DNA was evaluated via DNA extraction followed by real time PCR analysis in a single assay using the viral suspension A diluted or not. Each viral sample was split into two fractions. A fraction was treated at 97 °C for 45 min before viral DNA extraction in order to destroy viral capsids and served as a control. The other fraction was not heated. An aliquot from the unheated fraction was used as a control and was not treated with PMA, but the same amount of DMSO was added. To determine the optimal concentration of PMA, PMA treated heated and nonheated samples (undiluted and diluted to 1/10, 1/100, 1/1000 and 1/10 000) were incubated with 300 µM, 400 µM and 500 µM of PMA and exposed to halogen light for 10 min (PMA photo-activation).

As Ct levels are inversely proportional to the amount of the target DNA in the sample, the difference between the Ct values obtained for untreated and PMA-treated viral suspension by real time PCR (delta Ct) was calculated for each pair of samples as ΔCt = Ct viral suspension without PMA - Ct viral suspension with PMA. The proportion of undamaged capsids was estimated by the ratio of the number of OsHV-1 DNA copies in presence of PMA and the number of copies of OsHV-1 DNA without PMA.

2.5. Effect of heat on viral DNA detection

The viral suspension A was tested three times on separate runs and assays were carried out on the undiluted viral suspension (10^5 viral DNA copies/µL) and diluted to 1/10, 1/100, 1/1000 and 1/10 000. Samples were subjected or not to heat treatment (97 °C for 45 min) before viral DNA quantification using or not PMA. PMA was used at the final concentration of 500 µM.

2.6. Testing different viral suspensions

Four viral suspensions (A–D) prepared independently from each other were tested: suspensions A–C were stored at 4 °C during two months after preparation, the D suspension was stored at 4 °C three weeks before use. They were tested pure without PMA or with PMA at the final concentration of 500 µM. Negative control was achieved as previously described. For each condition (with or without PMA), viral DNA quantification was carried out three times in separate runs. The percentage of undamaged capsids was calculated as number of viral DNA copies per µL in the presence of PMA/number of viral DNA copies per µL without PMA x 100.

2.7. Oyster mortality and undamaged capsid number

Pacific oyster spat were first produced from genitors collected in the wild (Brittany) at the Ifremer's facilities (Argenton, France) and secondly grown at the Ifremer's facilities at Bouin (France). Less than one year old oysters were first placed out of water for 24 h at 22 °C and then 'anesthetized' during 4 h in a solution containing 7 % (w/v) magnesium chloride ($MgCl_2$; 50 g/L) in seawater (1 v)/distilled water (4 v) (Gagnaire et al., 2007; Schikorski et al., 2011a, b). They were then injected into the adductor muscle with 100 µL of the viral suspension A (undiluted or dilutions). After injection, each oyster was individually placed in a beaker containing 500 mL of filtered seawater UV treated, covered and aired by a bubbler. For the undiluted viral suspension and each tested dilution (1/10, 1/100, 1/1000 and 1/10 000), the number of oysters was equal to 10. The temperature of the water for all experiments was maintained at 22 °C. Three independent experiments were carried out. Although it is possible to reproduce the viral infection using different approaches, experimental infection by injection was chosen in this work, because it allows us to know precisely the volume injected and therefore also the quantity of DNA injected.

During these experiments, the amount of undamaged viral capsids injected to each series of 10 oysters was declined as follows: absence of undamaged capsids (1/10 000 dilution), 1 to 10 (1/1000 ditution), 11 to 200 (1/100 dilution), 201 to 1000 (1/10) and more than 6000 undamaged capsids (undiluted). Mortality was daily recorded for 7 days.

2.8. Data analysis

All analyses were conducted in the R studio software (version 3.3.2). Evalution of the best concentration of PMA was done with ggplot2. The Kruskal–Wallis test was carried out to determine whether significant differences exist between experimental treatments (optimizing PMA concentration, reproductibility test). Kaplan-Meier survival curves and the logrank test were used to characterize and compare survival between Pacific oyster conditions (packages survival, v2.39-5, and survminer, v. 0.4.3).

3. Results

3.1. Optimal PMA concentration needed to bind viral DNA

Results showed that for the lowest amounts of viral DNA (10^1 and 10^2 OsHV-1 DNA copies per µL), the highest final concentation of PMA (500 µM) yielded the highest differences in terms of values of Ct in comparison with the samples without PMA (Fig. 1A). The lowest amounts of viral DNA (10^1 and 10^2 copies OsHV-1 DNA per µL) yielded the highest ΔCt (Ct with PMA - Ct without PMA) for the highest final concentation of PMA (500 µM) (Fig. 1B). Additionally, the R^2 value obtained for this final PMA concentration was the highest and similar to those reported in absence of PMA treatment (Fig. 1A). Based on these first results, the PMA concentration 500 µM was selected for futher work. Additionally, no significant difference was observed between samples of virus suspension A without PMA and samples added with PMA (300 µM, 400 µM and 500 µM) in absence of photoactivation (data not shown).

3.2. Effect of heat treatment on viral DNA detection

For the nondiluted suspension A and the four tested viral suspension dilutions, no significant difference was reported for Ct values in absence of PMA between heated and non-heated samples (Fig. 2). For the five tested viral concentrations, Ct values were significantly ($p \leq 0{,}00047$) higher in samples treated with PMA in comparison with non-treated ones (Fig. 2). Finally, all samples tested after heating in presence of PMA showed significant ($p \leq 0{,}00047$) higher Ct values in comparison with samples treated with PMA in absence of heat treatement (Fig. 2). The results showed that PMA pre-treatment resulted in a significant decrease ($p \leq 0{,}00012$) in the detection of viral DNA by qPCR.

3.3. Testing four viral suspensions

The average viral DNA amount was 1.63 10^5, 1.97 10^5, 5.40 10^4 and 9.42 10^5 OsHV-1 DNA copies per µL for viral suspensions A, B, C and D, respectively, in absence of PMA and without heat treatement (Fig. 3A). After PMA pretreatment at the final concentration of 500 µM (in absence of heating), the average viral DNA amount yielded 1.42 10^4, 5.58 10^3, 2.14 10^3 and 1.29 10^5 OsHV-1 DNA copies per µL for viral suspensions A, B, C and D, respectively (Fig. 3A). Finally, the average viral DNA amount yielded 2.82 10^2, 4.77 10^2, 5.06 10^1 and 1.55 10^3 OsHV-1 DNA copies per µL for viral suspensions A, B, C and D, respectively, after heat inactivation at 97 °C for 45 min et PMA preatreatment (Fig. 3A).

For each viral suspension, the percentage of undamaged capsids was calculated as number of viral DNA copies per µL in the presence of PMA/number of viral DNA copies per µL without PMA x 100. The percentage of undamaged capsids was 8.71, 2.83, 3.96 and 13.68 for viral suspensions A, B, C and D, respectively (Fig 3B). After heating, the percentage of undamaged capsids varied from 0.3 to 0.74 in the different viral suspensions (Fig. 3B).

3.4. Oyster mortality and undamaged capsid number

At the end of the experiments (7 days post injection), main mortality percentages were 3.3 %, 14 %, 42.5 %, 53.3 % and 56.7 % corresponding to none undamaged capsid, 1 to 10, 11 to 200, 201 to 1000, and more than 6000 undamaged capsids, respectively (Fig. 4). The survival analysis using the Kaplan Meir approach allowed us to estimate the survival of the oysters according to the injected dose and to compare the tested doses. The log rank showed a strong significance ($p = 0{,}00018$).

4. Discussion

The lack of reliable virus cell culture systems makes it difficult to determine the integrity of OsHV-1 by only polymerase chain reaction (PCR) or real-time quantitative PCR (q-PCR). These methods can detect OsHV-1 genomes in a few hours, but they cannot distinguish between infectious and non-infectious virus particles. In this context, the first aim of the present study was to investigate the detection of undamaged viral capsids by combining PMA with q-PCR.

Propidium monoazide (PMA) is a membrane impermeant dye that

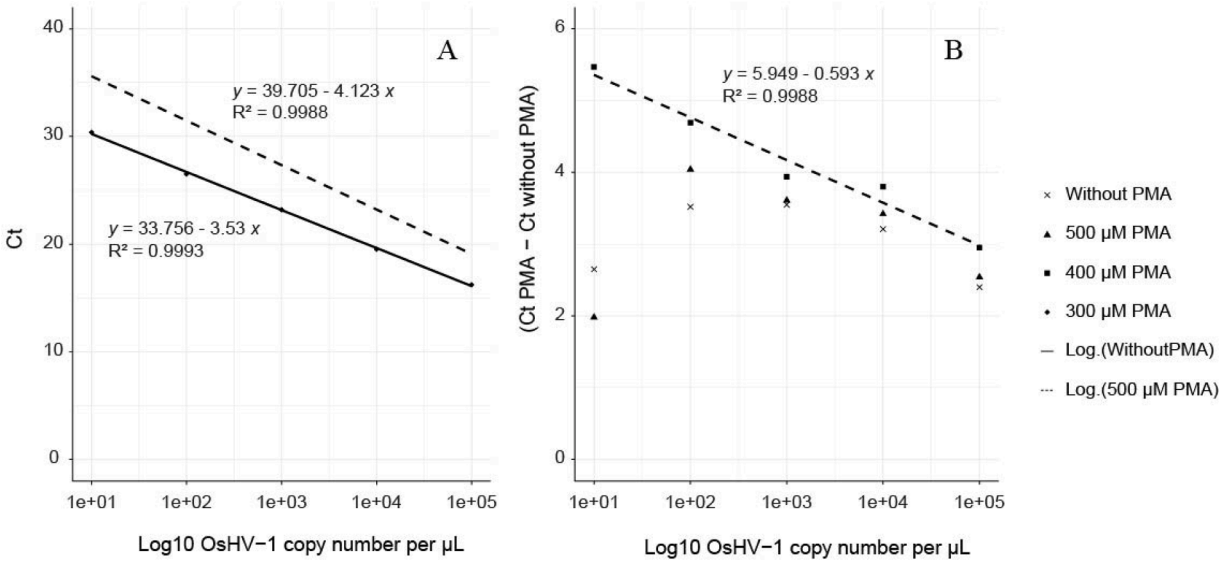

Fig. 1. Optimizing PMA concentration. The 1e+10^5 value on the X axis corresponds to the nondiluted viral suspension and the 1e+10^1 value to the 1/10 000 dilution. *1*A-Test of 3 concentrations of PMA on the virus suspension A at different dilutions (nondiluted to 1/10 000). 1B- Test of different concentrations of PMA and different dilutions of the virus suspension A expressed in delta Ct (Ct with PMA - Ct without PMA).

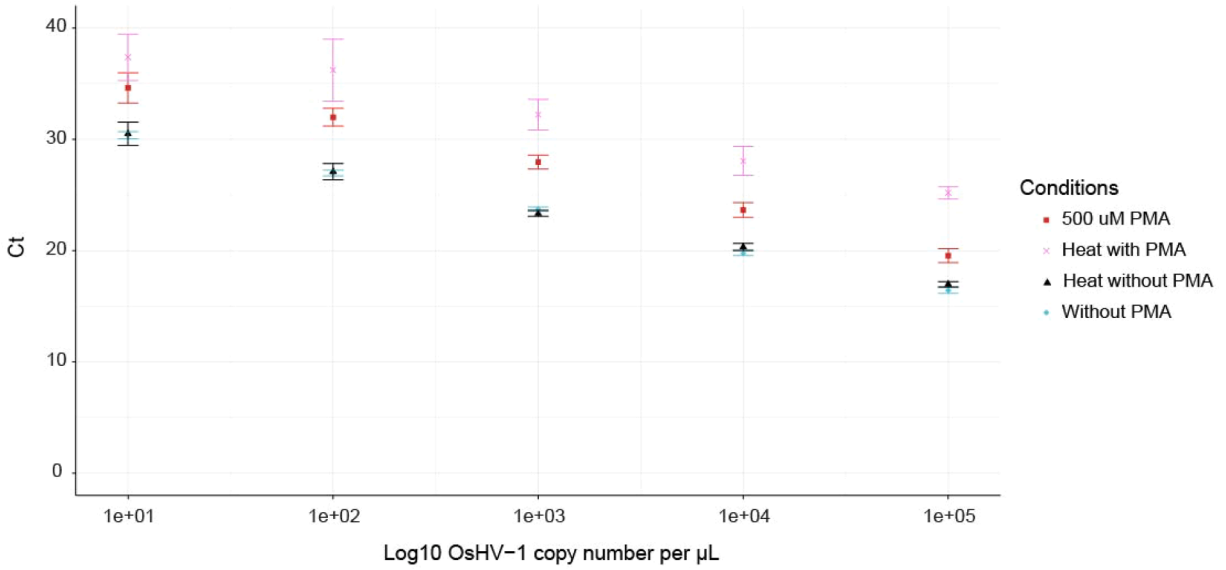

Fig. 2. Viral DNA detection in the suspension A at different dilutions with or without PMA (500 µM) and with or without exposure to 97 °C for 45 min. The 1e+10^5 value on the X axis corresponds to the nondiluted viral suspension and the 1e+10^1 value to the 1/10 000 dilution.

selectively penetrates cells with compromised membranes. Inside the cells, the dye can intercalate into nucleic acids and inhibit PCR amplification. The PMA binding mechanism has previously been used to differentiate between live and dead microorganisms including bacteria, fungi and parasites (Brescia et al., 2009; Nocker et al., 2009; Pan and Breidt, 2007; Rousseau et al., 2019; Vesper et al., 2008), or between infectious and non-infectious viruses (Canh et al., 2021, 2022; Coudray-Meunier et al., 2013; Fongaro et al., 2016; Fittipaldi et al., 2010; Kim and Ko, 2012; Lee et al., 2015; Leifels et al., 2015, 2021; Liang et al., 2021; Moreno et al., 2015; Parshionikar et al., 2010; Prevost et al., 2016; Sanchez et al., 2012). Thus, PMA-qPCR could potentially be used to measure the integrity of OsHV-1 particles. A set of parameters that may affect the efficiency of PMA-qPCR must be taken into account as the optimal conditions for PMA-qPCR may vary with microbe species (Fittipaldi et al., 2010). To assess whether PMA pretreatment is a suitable approach to inhibit DNA amplification from damaged OsHV-1, virus DNA was measured by qPCR and PMA-qPCR. Although the integrity of viruses belonging to different families has previously been investigated, to our knowledge, there is no previous published study that investigated the effectiveness of PMA for quantifying undamaged capsids of vertebrate herpesviruses.

The three different concentrations of PMA were selected based on the existing body of literature (Bae and Wuertz, 2009; Brescia et al., 2009; Varma et al., 2009). Final PMA concentrations varied from 10 µM to 1000 µM in reported studies. As an example, the PMA solution was added at a final concentration of 100 µM to discriminate infectious bacteriophage T4 virus by PMA real-time PCR (Fittipaldi et al., 2010). Zeng et al. (2016) also reported that the highest percentage of dead cells of *E. coli* O157:H7 was observed when a 100 µM concentration of PMA was used. However, Karim et al. (2015) used a final concentration of PMA adjusted to 348 µM for detecting infectious enterovirus and norovirus. Additionally, Randazo et al. (2016) evaluated conventional

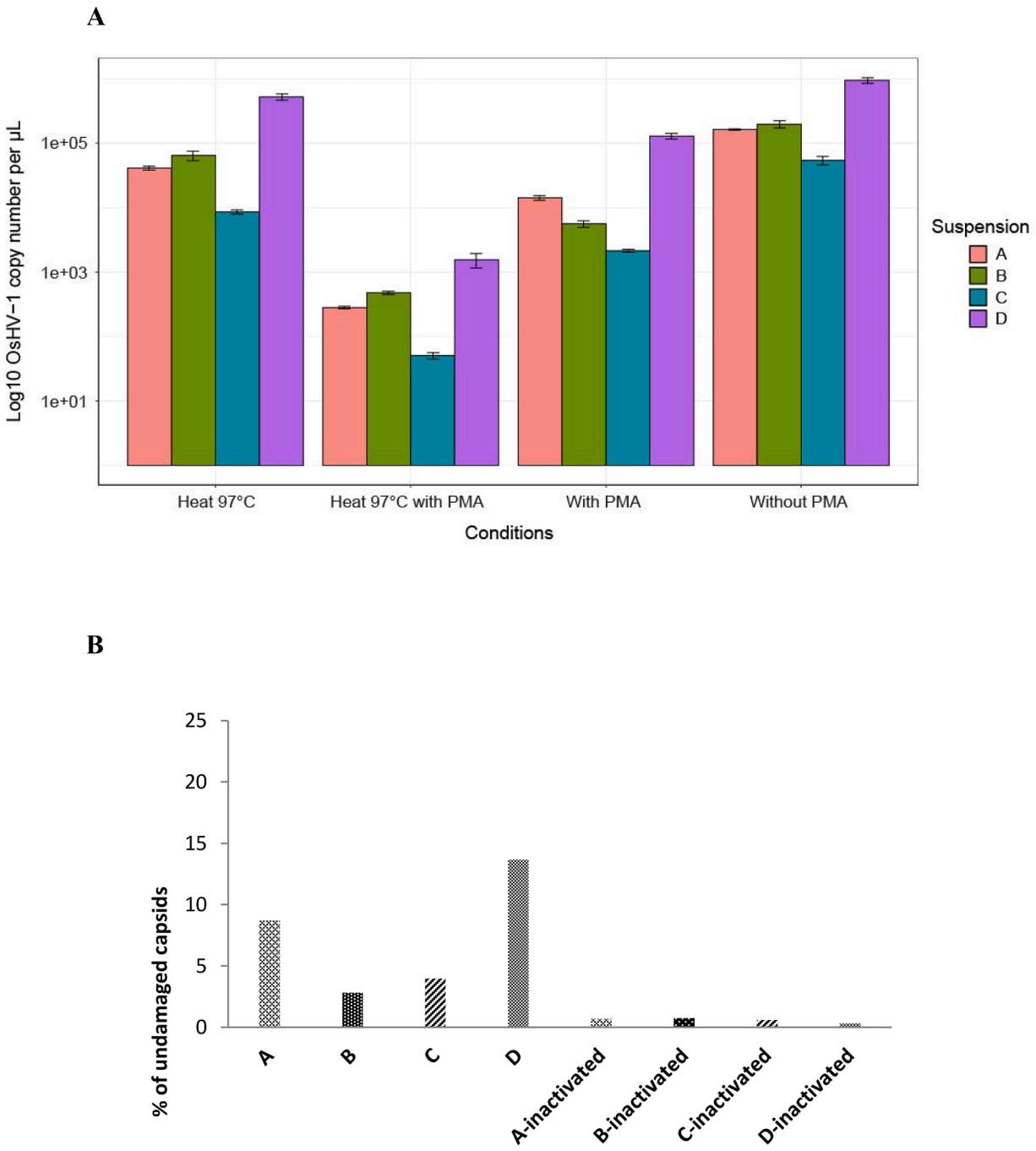

Fig. 3. 3A-Viral DNA detecton in viral suspensions A–D undiluted with or without PMA (500 µM) and with or without heat treated. 3B- Calculation of the number of intact capsids (expressed as a percentage) in the four tested viral suspension.

photoactivatable dyes (PMA and ethidium monoazide, EMA) and newly developed ones (i. e. PMAxx and PEMAX) for the discrimination between infectious and thermally inactivated NoV genogroup I (GI) and II (GII). These authors reported that PMAxx was the best photoactivatable dye using a 50 µM final concentration for assessing norovirus infectivity. In this context, it would be of interest to evaluate PMAxx to discriminate native and thermally inactivated OsHV-1 suspensions.

PMA could penetrate the OsHV-1 capsids and bind to viral DNA as a result of the denaturation of capsid proteins at 97 °C for 45 min. Parshionikar et al. (2010) used propidium monoazide in reverse transcriptase PCR to distinguish between infectious and non-infectious enteric viruses in water samples. These authors investigated virus inactivation by treatment with heat (72 °C, 37 °C, and 19 °C) or hypochlorite on coxsackievirus, poliovirus, echovirus, and Norwalk virus. Parshionikar et al. (2010) reported that the use of PMA did not interfere with detection of native viruses but prevented detection of noninfectious or inactivated viruses that were rendered noninfectious or inactivated by treatment at 72 °C and 37 °C. However, PMA-RT-PCR was unable to prevent detection of enteroviruses that were rendered noninfectious by treatment at 19 °C. Additionally, some differences were also reported depending of tested viruses. Although poliovirus after treatment at 37 °C was undetectable by qRT-PCR including PMA pretreatment, Norwalk virus remained detectable suggesting differences in terms of stability to heat treatement depending of the virus nature (Parshionikar et al., 2010). Fittipaldi et al. (2010) tested different inactivation treatments including heat (85 °C) and proteolysis in order

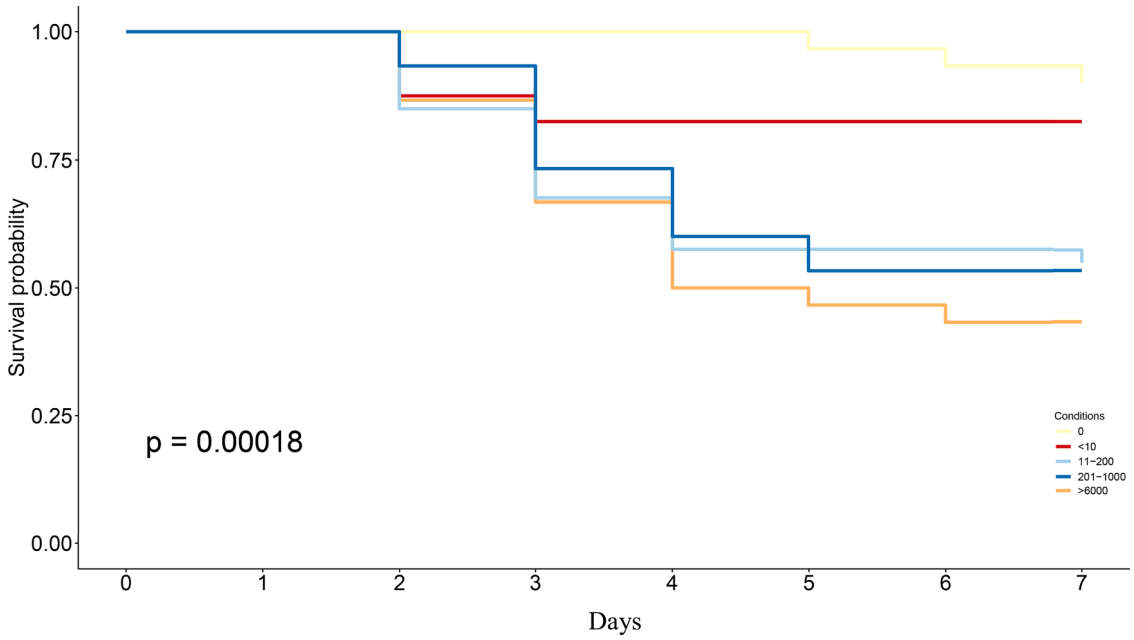

Fig. 4. Kaplan-Meier survival curves of Pacific oysters injected with the OsHV-1 suspension A from three different experiments with Log-rank ($p = 0.00018$). The amount of estimated undamaged viral capsids injected to each series of 10 oysters was declined as follows: absence of undamaged capsids (1/10 000 dilution), 1 to 10 (1/1000 ditution), 11 to 200 (1/100 dilution), 201 to 1000 (1/10) and more than 6000 undamaged capsids (undiluted).

to discriminate infectious bacteriophage T4 virus by PMA real-time PCR. Athough after these inactivation treatments, the PMA pre-treatment approach appeared not appropriate for differentiating infectious viruses, a higher inactivation temperature (110 °C) and PMA pre-treatment did allow differentiation of non-infectious viruses from infectious ones (Fittipaldi et al., 2010).

Additionally, as PMA is considered effective only with viruses when inactivation leads to capsid damage, the heat treatment was selected in the present study for OsHV-1 inactivation. Chlorine and UV treatments damage first viral nucleic acid, though protein damage can occur at high concentrations or doses (Li et al., 2002; Eischeid and Linden, 2011; Karim et al., 2015). Eischeid and Linden (2011) examined UV damage on adenovirus proteins and reported that medium pressure UV was more effective at damaging viral proteins at high UV doses, though low presure UV caused some damage as well. PMA RT-PCR could only differentiate between infectious and chlorine inactivated MNV-1 when MNV-1 was inactivated using a high chlorine concentration suggesting that a higher chlorine concentration is required for MNV-1 capsid damage and subsequent PMA entrance and binding (Karim et al., 2015).

After PMA pretreatment at the final concentration of 500 μM (in absence of heating), the average viral DNA amount yielded from 2.14 10^3 to 1.29 10^5 OsHV-1 DNA copies per μL for the different viral suspensions demonstrating a significant decrease in comparison with the results obtained in absence of PMA. These results suggested that in absence of virus denaturation treatment a large number of damaged capsids and/or free viral DNA were already present in the tested viral suspensions. Thus, PCR OsHV-1 detection by PCR overestimated infectious viral targets. Moreover, the percentage of undamaged capsids appeared quite limited in the four viral suspensions (from 2.8 to 13.6 %). Finally, virus heating at 97°C for 45 min was highly effective for denaturation of OsHV-1 particles.

Three experimental viral infection assays were carried out using different dilutions of the viral suspension A in order to estimate a viral LD50. It is important to notice that OsHV-1 has a viral envelope covering its capsid that is used to help viruses enter host cells, and most enveloped viruses are dependent on the envelope for their infectivity (Mahy, 1998). Thus, the viral envelope can be damaged inactivating the virion but leaving its capsid uncompromised. Estimating a number of uncompromised OsHV-1 capsids overestimates the number of infectious particules as some undamaged capsids could present a damaged envelop.

A percentage of 53.3 % of mortality was reported 7 days post infection for Pacific oysters receiving from 201 to 1000 undamaged capsids. As the estimated number of uncompromised OsHV-1 capsids by PMA PCR overestimates the number of infectious particules, the LD50 could be less than 201 to 1000 viral particles. Moreover, the injection of 11 to 200 undamaged capsids also induced some mortality (42.5 %) 7 days post infection. OsHV-1 appeared thus as a highly pathogenic virus. Schikorsky et al. (2011a, b) were the first authors to describe effective induction of OsHV-1 infection in *C. gigas* spat by injection and cohabitation in experimental conditions. Significant mortality (more than 60 % 7 days post injection) was induced after injection of 100 μL of a tissue homogenate containing 2 10^2 viral DNA copies per μL (2 10^4 viral DNA copies per oyster). If we consider based on the results of our study that around 10 % of viral DNA copies corresponded to undamaged capsids in the viral suspensions used by Schikorsky et al. (2011a), around 2000 uncompromised capsids were related to 60 % mortality. This extrapolation is open to discussion because the proportion of DNA present in capsids in an infectious format could be different between preparation obtained from oysters at different stages of infection. A dose-response relationship between viral DNA concentration in the inoculum and the percentage of mortality was also investigated through experimental trials by Paul-Pont et al. (2015). These authors reported the identification of an appropriate inoculum concentration (5 10^3 copies of viral DNA ml^{-1}) leading to slow mortality kinetics and intermediate cumulative mortality. Around 500 uncompromised capsids might thus induced mortality considering that 10 % of viral DNA copies corresponded to undamaged capsids in this appropriate inoculum. As the number of undamalged OsHV-1 capsids could varied between different tissue homogenates, estimating this number using PMA qPCR appears as a helpful prerequisite prior performing experimental trials. Corbeil et al. (2012) reported that abalone herpesvirus (AbHV) was highly pathogenic in abalone based on dose-response curves, suggesting that relatively few virus particles were required to induce disease and related lesions. However, these authors noticed that qPCR used for experimental assays did not allow precise quantification of infectious viral particles present.

Finally, defining a virus LD50 remains a complexe question as a genetic basis of a better resistance to OsHV-1 infection has been demonstrated through the production of *C. gigas* family lines presenting variable susceptibility to the infection and differential survival rates (Segarra et al., 2014a, 2014b; Degremont et al., 2015; Azema et al., 2016, 2017).

As a main result of the present study, the combination of PMA coupled to real-time PCR is a promising tool for prediction of OsHV-1 infectivity in absence of currently reliable cell culture techniques. This approach can be usefull to investigate OsHV-1 status in marine molluscs including Pacific oysters and in the marine environment. The developped PMA PCR technique is not intended to replace the quantification of viral DNA by qPCR, but it does make it possible to give a form of biological meaning to the detection of this DNA.

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CRediT authorship contribution statement

Tristan Renault: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nicole Faury:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Benjamin Morga:** Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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