RAPID AND SENSITIVE DETECTION OF OSTREID HERPESVIRUS (OsHV-1) IN OYSTER SAMPLES BY REAL-TIME PCR

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ABSTRACT

Herpes and herpes-like virus infections have been reported in various marine mollusc species associated with high mortality rates. Specific diagnostic tools have been developed based on conventional PCR techniques or in situ hybridisation. We developed a real-time PCR assay for rapid, sensitive and quantitative detection of OsHV-1. The new assay utilised SYBR® Green chemistry with specific primers C9/C10, B3/B4, Gp4/Gp7 targeting three different regions. The assay was applied successfully to rapid diagnosis (100min) of OsHV-1 in different developmental stages and tissue samples of Crassostrea gigas. The quantitative data that will emerge in future using the new assay will illuminate aspects of pathogenesis, in particular the viral loads in asymptomatic oysters and the kinetics of infection in specific target tissues.

INTRODUCTION

Herpesvirus infections have been associated with high mortality in the larvae and spat of oyster, and other species (clams, scallop, abalone). The virus isolated from infected C. gigas larvae has been classified as a member of the family Herpesviridae under the species name Ostreid herpesvirus 1 (OsHV-1). Routine detection of OsHV-1 is performed by conventional PCR, (Renault and Arzul, 2001; Friedman et al., 2005). In addition, a semi-quantitative assay has been developed based on competitive PCR (Renault et al., 2004). Real-time PCR would overcome the disadvantages of conventional PCR and render key epidemiological questions tractable, with considerable practical outcome. We present the development of a new diagnostic tool based on SYBR® Green chemistry real-time PCR method to detect and quantitate OsHV-1 (and OsHV-1 variant) in C. gigas.

MATERIAL & METHOD

REAL-TIME PCR : ***Three sets of OsHV-1 specific primers were assessed. The primer pairs B_4/B_3, C_9/C_{10} and Gp_4/Gp_7 yielded PCR products of 207, 197 and 85 bp, respectively. Amplification reactions were performed using a Mx3000 Thermocycler sequence detector (Stratagene) with 96 microwell plates. Each well (25 µL) contained 5 µL of extracted DNA (tested sample) or OsHV-1 genomic DNA (positive control, standard), 12.5 µL of Fullvelocity® Master Mix (hot start DNA polymerase, Stratagene), 2.5 µL of each diluted primer (2µM) and 2.5 µL of distilled water. Thermal cycle conditions included amplification steps plus melting temperature (Tm) curve step. All Real-time PCR tests were performed in triplicate. The real-time PCR was based on SYBR® Green chemistry and absolute quantitation of copies of OsHV-1 DNA (copies/µL) using a standard curve based on serial dilutions of purified genomic viral DNA.***

PCR : ***Single PCR were performed using C2/C6 primer set according Renault et al, 2000.***

OYSTER SAMPLES : ***Samples were C. gigas from various locations in France. DNA was extracted using a commercial kit (QIAgen - Qiamp tissue mini kit®). Prior to analysis, sample DNA concentration was adjusted to 4 ng /µL.***

RESULTS

PRIMER SETS VALIDATION *: (fig1-2)*

- *Primer pairs, C9/C10, B3/B4, Gp4/Gp7, were validated, yielded :*

PCR Efficiency ***90%< E >110% ; R^2~ 0,99 ; Slope -3,5 < S > -3,3****.*

SPECIFICITY *: (fig3)*

- *Melting curves of the amplified fragments generated by primer sets presented a single peak: B_3/B_4 Tm=79,5 °C; Gp_4/Gp_7 Tm=75,6 °C; C_9/C_{10} Tm =75,7 °C.*

DYNAMIC RANGE *(C9/C10) : (fig4)*

- ***10 copies to 5.10^6 DNA copies / µL.***

COMPARATIVE SENSITIVITY *: (fig5 & Table1)*

- *Analytical sensitivity reached* ***2 viral DNA copies/µL****. Real-time PCR assay was one hundred fold more sensitive than single PCR (C2/C6 primers).*

OsHV-1 DIAGNOSIS *: (fig7-8)*

- *Detection of OsHV-1 DNA and associated viral load was assessed in uninfected and infected batches of larvae and spat.*

CONCLUSIONS

- ***This PCR quantitative method was successfully used to detect and quantify OsHV-1 DNA during productive infections in oyster larvae, seed and spat, included during latency period.***
- ***The increase of sensitivity allowed to detect more positive samples than routine PCR, namely in case of asymptomatic samples (no mortality).***
- ***Quantitative data will help in better understanding of the disease in particular the kinetics of infection in specific target tissues.***
- ***All the advantages of this new diagnostic tool should improve better sanitary management.***

References :

Friedman, C.S., Estes, R.M., Stokes, N.A., Burge, C.A., Hargove, J.S., Barber, B.J., Elston, R.A., Burreson, E.M., Reece, K.S., 2005. Herpes virus in juvenile Pacific oysters Crassostrea gigas from Tomales Bay, California, coincides with summer mortality episodes. Dis. Aquat. Org. 63, 33-41

Renault, T., Le Deuff, R-M., Lipart, C. & Delsert, C., 2000. Development of a PCR procedure for the detection of a herpes-like virus infecting oysters in France. J Virol Methods 88, 41-50.

Renault T. and Arzul I. 2001. Herpes-like virus infections in hatchery-reared bivalve larvae in europe: specific viral DNA detection by PCR. J. Fish Dis 24, 161-167.

Renault T, Arzul I, Lipart C., 2004. Developement and use of an internal standard for oyster herpesvirus detection by PCR. J Virol Methods 121:17–23.

RESULTS: *SYBR Green real-time PCR assay*

Fig1: OsHV-1 Standard curve using C9/C10 primers

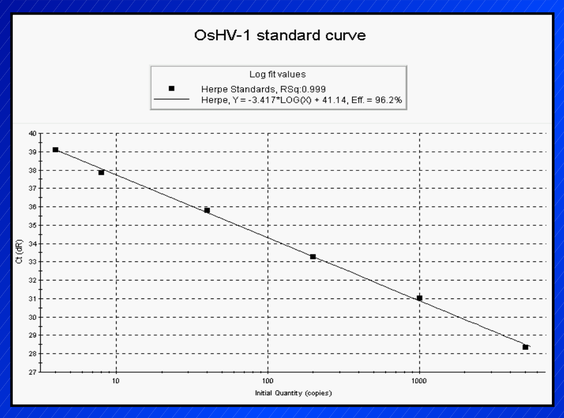
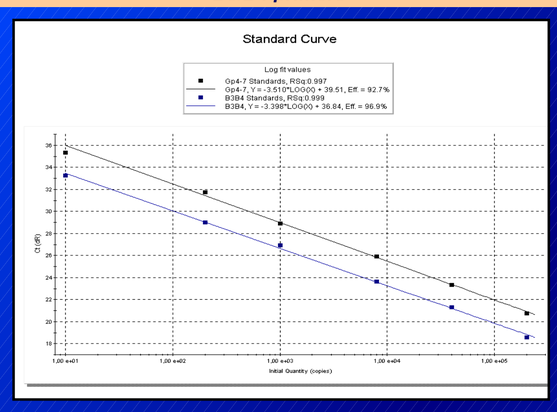


Fig2: OsHV-1 Standard curve using Gp4/Gp7 and B3/B4 primers



RESULTS : *specificity, dynamic range*

Fig3: Specific melting curve

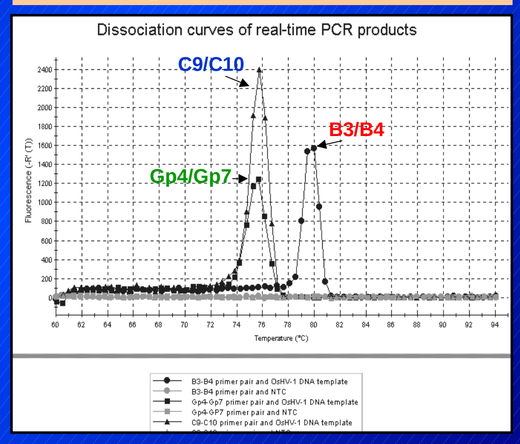
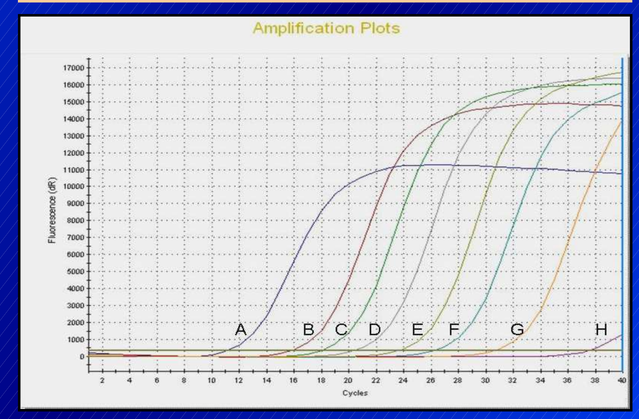


Fig4: Dynamic range using C9/C10 primers

A, 5 x 10^6 copies/µL; B, 2 x 10^5 copies; C, 4 x 10^4 copies; D, 8 x 10^3 copies; E, 1 x 10^3 copies; F, 2 x 10^2 copies; G, 1 x 10^1 copies; H, no template DNA, or negative control (NTC)



RESULTS : *sensitivity and OsHV-1 Diagnosis*

Table1: ***Comparison of some samples analysed using SYBR Green PCR and Conventional PCR***

Batches or samples ref.	Animal size	Tissue analysed	Abnormal mortalities in batches	SYBR Green PCR results (viral DNA copy number / mg)	Conventional PCR using C_2/C_6 primer pair
1	850 µm	*in toto*	Yes	6.28E+03	-
2	850 µm	*in toto*	Yes	7.86E+03	-
3	400 µm	*in toto*	Yes	2.10E+06	+
4	> 200 µm	*in toto*	Yes	1.49E+06	+
5	> 120 µm	*in toto*	Yes	8.81E+06	+
6	> 140 µm	*in toto*	Yes	1.38E+07	+
7	400 µm	*in toto*	Yes	3.73E+05	+
8	1.5 mm	*in toto*	Yes	1.7E+04	+
9	180 µm	*in toto*	No	4.51E+01	-
10	140 µm	*in toto*	Yes	2.62E+07	+
11	400 µm	*in toto*	Yes	1.75E+05	+
12	> 200 µm	*in toto*	No	4.10E+01	-
13	> 140 µm	*in toto*	Yes	5.38E+07	+
14	60 µm	*in toto*	No	5.0E+01	-
15	60 µm	*in toto*	No	0: No CT	-
16	> 3 cm	Mantle	No	0: No CT	-
17	> 3 cm	Mantle	No	6.5E+02	-
18	> 3 cm	Mantle	Yes	2.31E+07	+
19	> 3 cm	Mantle	Yes	1.21E+07	+
20	> 3 cm	Mantle	Yes	3.1E+06	+
21	> 3 cm	Mantle	No	9.9E+02	-
22	> 3 cm	Mantle	No	5.0E+02	-
23	> 3 cm	Mantle	No	0: No CT	-
24	> 3 cm	Mantle	Yes	6.3E+06	+
25	> 3 cm	Mantle	Yes	4.2E+06	+

Fig5: Comparative Sensitivity PCR versus Real-time PCR

Conventional PCR using the C_2/C_6 primers, real-time PCR using the C_9/C_{10} primers with OsHV-1 dilutions (copy/µL). Lane 1: size markers. Lanes 2-8: PCR products from conventional PCR (710 bp). Lanes 15-20: real-time PCR products, expected amplicon size 197 bp.

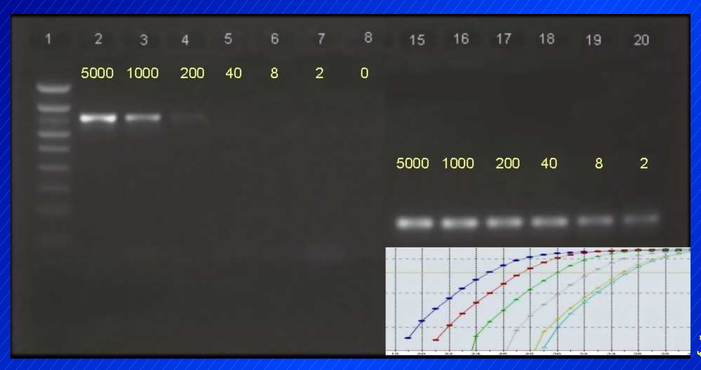


Fig7: Viral load kinetic during a larval OsHV-1 outbreak

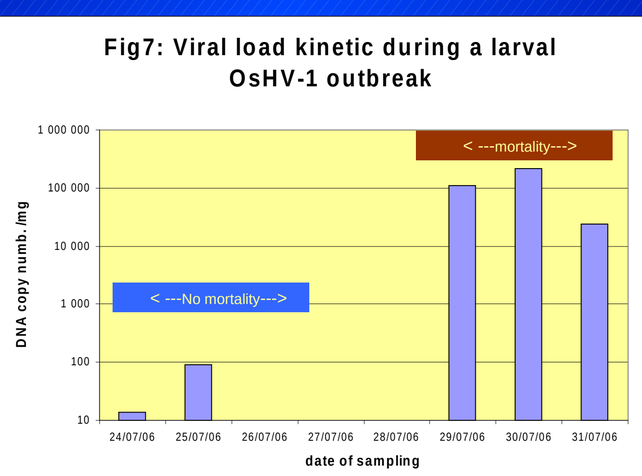
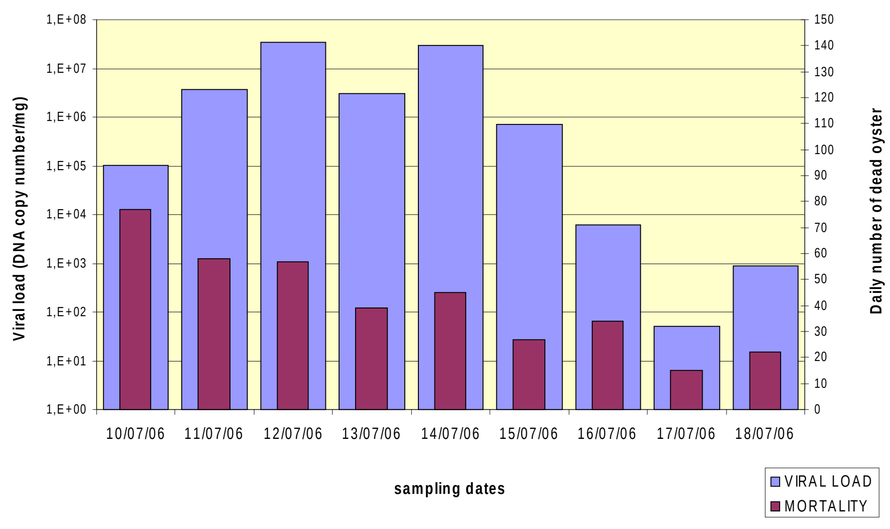


Fig8: Viral load kinetics in alive oysters during an herpesvirus outbreak and associated mortality in *C. gigas* , 6 month old



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