Aquaculture 354–355 (2012) 64–67

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Influence of temperature and exposure time on the infectivity of Bohle iridovirus, a ranavirus

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ARTICLE INFO

Article history:
Received 12 March 2012
Accepted 5 April 2012
Available online 13 April 2012

Keywords:
Ranavirus
BIV
Temperature
Infectivity

ABSTRACT

This study examined the functional temperature range of a ranavirus outside host cells over increasing temperatures and exposure times and subsequently tested infectivity in cell culture. Initially, cell susceptibility was determined by incubating Bohle iridovirus (BIV) at 30 °C, 40 °C, 50 °C and 60 °C for 5, 30 and 60 min and subsequently titrating samples in one epithelioma papulosum cyprinid (EPC) and two Bluegill fry 2 (BF2) lineages at 28 °C. Titres obtained in the three cell lines were similar and EPC cells were subsequently used to further investigate ranavirus infectivity with two degree increments in temperature between 40 °C and 60 °C for 5, 30 and 60 min. The rate of inactivation was found to be dependent on temperature and time of exposure. Bohle iridovirus could replicate in EPC cells following exposure to most temperatures and prolonged time, but titers were reduced as temperature and time of exposure increased. Viral titres were greatest (10^8 $TCID_{50}$/ml) after exposure to 30 °C and declined with increasing time of exposure and increasing temperature. Declines in BIV infectivity were largely between 40 °C (10^8 $TCID_{50}$/ml) and 44 °C (10^5 $TCID_{50}$/ml at 5 and 30 min and $10^{3.5}$ $TCID_{50}$/ml at 60 min) and secondly at temperatures greater than 52 °C (from $10^{3.5}$ $TCID_{50}$/ml and approaching zero with increasing temperature and time). Treatment at 58 °C for 60 min and 60 °C for 30 and 60 min resulted in complete loss of BIV infectivity. The results from this study show that ranavirus can withstand much higher temperatures than previously thought, which is fundamental for understanding ranavirus epidemiology, indirect transmission dynamics and for biosecurity purposes.

1. Introduction

Ranaviruses have a broad host range including fish, reptiles and amphibians (Ariel et al., 2009a, 2010; De Voe et al., 2004; Johnson et al., 2008; Langdon et al., 1988; OIE, 2008), with temperatures appearing to play an important role in their virulence (Ariel and Jensen, 2009; Rojas et al., 2005; Whittington and Reddacliff, 1995). *In vitro* trials tested the replication of a panel of ranaviruses in a selection of fish cell lines at different temperatures and found that individual isolates have distinct preference for both cell line and incubation temperature (Crane et al., 2005; Ariel et al., 2009b). Speare and Smith (1992) reported that the ranavirus, Bohle iridovirus (BIV) grew to high titers in several fish cell lines at temperatures between 20 °C and 30 °C, but did not detect replication above 34 °C, which supports the findings for other ranaviruses (Granoff et al., 1965; Gravell and Granoff, 1970). However, exposure to 37 °C for six hours did not decrease the titre of the virus, which indicates that even if it does not replicate at this temperature, it is still viable (Speare and Smith, 1992). In fact, there is a paradigm in the literature that iridoviruses do not replicate above 32 °C (Chinchar et al., 2009; for a minireview see Moody, 1992). The issue with all of these systems is that they are multi-factorial and it is difficult to separate the ability of the virus to replicate from the ability of the propagation system (here cell lines) to support the viral replication at temperatures outside the cells preferred range. The activity level of the immune system in the live poikilothermic host is also temperature dependent and may further confound the picture (Bly and Clem, 1992; Carey et al., 1999; Le Morvan et al., 1998; Wright and Cooper, 1981). While the interactions between the virus and the host cells or host animal are important from the point of view of virulence, pathogenicity, laboratory diagnosis and transmission trials it is also important to separate the two systems in order to understand aspects of the epidemiology of the virus outside a host system and thereby enable sound biosecurity, control, containment and eradication decisions to be made. This study defines the functional temperature range that a ranavirus can tolerate outside the host by testing its ability to replicate in cell culture at normal culture conditions after exposure to various temperatures and exposure times.

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doi:10.1016/j.aquaculture.2012.04.006

2. Material and methods

2.1. Cell cultures

Three fish cell lineages: two lineages of Bluegill fry (BF2a and BF2b) cells (Wolf et al., 1966) and one lineage of epithelioma papulosum cyprini (EPC) cells (Fijan et al., 1983) in use in the virology laboratory at the School of Veterinary and Biomedical Sciences, James Cook University were initially tested for suitability. The cells were cultured in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 5% foetal bovine serum (FBS) in either 25 cm^2 sterile, non-vented tissue culture flasks (Sarstedt) for viral propagation or in 96-well flat bottom tissue culture plates (Sarstedt) for the viral titrations and incubated at 28 °C in a CO_2 rich environment.

2.2. Viral stock preparation

Bohle iridovirus (BIV) was originally isolated from ornate borrowing frogs (*Limnodynastes ornatus*) in the local suburb of Bohle (Speare and Smith, 1992) and was propagated in BF2 cells at 28 °C and stored at −80 °C. Stocks of BIV, defrosted at room-temperature, were inoculated onto cell cultures at a volume of 20 µl per flask of the three respective cell lineages. Fresh growth medium was then added to each flask. Cells were incubated at 28 °C and observed daily for cytopathic effect (CPE). When CPE was observed throughout the entire monolayer (rounding and lifting of cells from the bottom of the flask), flasks were freeze-thawed three times to release BIV from the cells. The cell culture medium including cell debris, was then transferred to sterile 10 ml, screw cap centrifuge tubes and centrifuged at 4500 *g* for 5 min using Eppendorf Centrifuge 5804 to pellet cell debris. Supernatant containing BIV was aliquoted into cryovials and stored at −80 °C until required.

2.3. Testing effect of temperature and exposure times on viral titre

Initially, the effect of temperature and exposure time on infectivity of BIV was tested in all three lineages at 10 °C intervals to compare cell lineage susceptibility. Subsequently, one lineage was selected to be tested at 2 °C increments.

Confluent monolayers of each lineage were prepared in 96-well flat bottom tissue culture plates with 100 µl medium (DMEM, 5% FBS) per well. Fifty µl aliquots of BIV were exposed to 30 °C, 40 °C, 50 °C and 60 °C for 5, 30 or 60 minutes in an Eppendorf Mastercycler Gradient Thermocycler (Eppendorf, Germany).

2.4. Titrations

Serial 10-fold dilutions (10^1 to 10^8) of each aliquot were prepared in growth medium. Fifty microliters of each BIV dilution were added to four wells of the 96-well tissue culture plates, starting from the highest dilution to the lowest. Fifty µl of growth medium was added to control wells. Cells were incubated at 28 °C in a CO_2 rich incubator, and observed daily for cytopathic effect (CPE) for seven days. The viral titre in each of the aliquots was determined using the Reed and Muench method (Reed and Muench, 1938) for calculation of 50% tissue culture infectious dose ($TCID_{50}$) on day seven following inoculation.

Additional BIV aliquots (Section 2.2) were then exposed to two degree increments between 40 °C and 60 °C for 5, 30 or 60 minutes and the titre determined in EPC cells as described above.

3. Results

3.1. Cell line susceptibility

Viral replication, detected as CPE, was observed in all cell lineages at most temperatures and exposure times (Table 1). Viral replication, reaching a maximum 10^8 $TCID_{50}$/ml was observed in BF2b and EPC cell lineages with BIV incubated at 30 °C and 40 °C for 5, 30 and 60 minutes. Inconsistencies were observed within the BF2a cell lineage where maximum titres were only observed in cells inoculated with BIV incubated at 40 °C for 5 and 30 minutes (Table 1).

Virus titres ranged between 10^2 $TCID_{50}$/ml and 10^3 $TCID_{50}$ /ml (Table 1), following incubation with BIV exposed to 50 °C for 5, 30 and 60 min. Cytopathic effect was observed in all cell lineages inoculated with BIV incubated at 60 °C for 5 min. However, CPE was not observed in any cell lineage inoculated with BIV incubated at 60 °C for 30 or 60 min. Control cells did not exhibit signs of CPE. Overall, results were similar for all three lineages.

Table 1
Titre ($TCID_{50}$/ml) of BIV in BF-2 (lineages a and b) and EPC cells after exposure of the virus to 30 °C, 40 °C, 50 °C and 60 °C for 5, 30 or 60 min. $TCID_{50}$ titres in bold represent observed differences between cell lineages.

Cell type	Time	Temperature			
		30 °C	40 °C	50 °C	60 °C
BF2-a	5 min	**10^5**	10^8	**10^3**	$10^{1.5}$
	30 min	**$10^{7.33}$**	10^8	$10^{2.5}$	N/A
	60 min	**$10^{5.66}$**	**10^7**	$10^{2.5}$	N/A
BF2-b	5 min	10^8	10^8	**10^2**	$10^{1.5}$
	30 min	10^8	10^8	**10^3**	N/A
	60 min	10^8	10^8	$10^{2.5}$	N/A
EPC	5 min	10^8	10^8	$10^{2.5}$	$10^{1.5}$
	30 min	10^8	10^8	$10^{2.5}$	N/A
	60 min	10^8	10^8	$10^{2.5}$	N/A

3.2. Propagation of ranavirus in cell culture following BIV exposure to temperature ranges between 40 °C and 60 °C in 2 °C increments

Epithelioma papulosum cyprini cells were chosen for this experiment due to mixed results obtained from the BF2 cell lineages, the relative ease of determining CPE in EPC cells and a recommendation arising from previous studies (Ariel et al., 2009b). Viral titre was of a maximum $TCID_{50}$ of 10^8/ml in EPC cells inoculated with BIV incubated at 40 °C for 5, 30 and 60 min. Viral titres were generally greatest at the 5 minute BIV incubation, followed by 30 min and 60 min for each two degree increase in temperature (Fig. 1). Viral titres declined rapidly between 40 °C and 44 °C for all incubation times. Viral titres appeared to plateau between 46 °C and 52 °C for 30 and 60 min incubation time and decreased slightly when BIV was incubated at the same temperatures for only 5 min. Viral titres decreased substantially for all incubation periods for temperatures above 52 °C (Fig. 1). Ranavirus infectivity was inhibited when exposed to 60 °C for 30 and 60 minutes (Fig. 1). CPE was not observed within control cells.

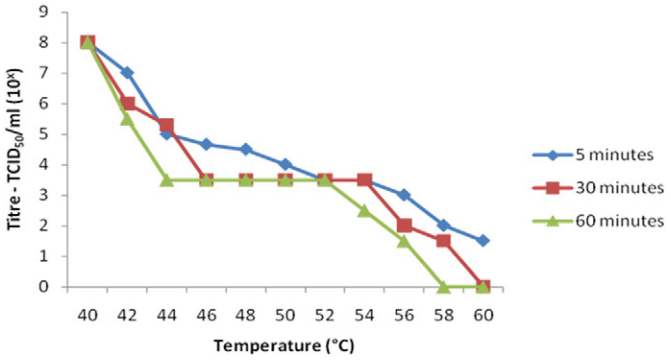

Fig. 1. $TCID_{50}$ of Bohle iridivirus (BIV) in epithelioma papilloma cyprini (EPC) cells following exposure to temperatures between 40 °C and 60 °C for 5, 30 and 60 min.