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

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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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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.

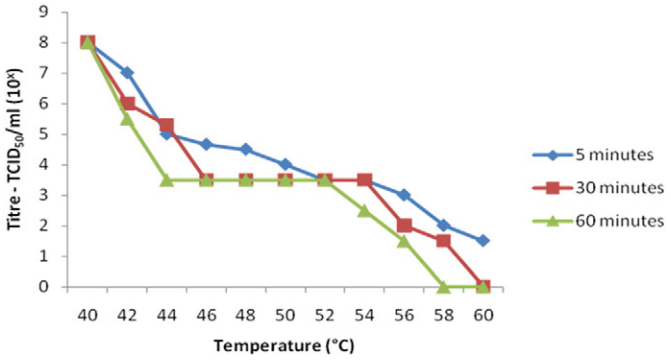

Fig. 1. $TCID_{50}$ of Bohle iridivirus (BIV) in epithelioma papilloma cyprini (EPC) cells following exposure to temperatures between 40 °C and 60 °C for 5, 30 and 60 min.

4. Discussion

Effects of temperature on ranaviral growth in cell culture has been described in detail (Ariel et al., 2009b), but the effect of temperature on pathogen infectivity outside the host is poorly understood. In this study, we explored the effects of temperature and time of exposure on the functional temperature range of a ranavirus outside host cells. Increasing temperature and time of exposure influenced ranavirus infectivity in all three cell lineages tested. Aliquots of the same viral sample showed considerable differences in titers depending on the incubation time and temperature. EPC and BF2b cell lineages performed well and the titres obtained in the two cell lines were similar. However, the BF2a cells consistently produced lower titres than the other cell lineages at 30 °C and 40 °C. The passage of cell cultures may adversely influence cell susceptibility to viral infection due to increased chances for mutations and subsequently changes in cell characteristics and susceptibility to virus over time (Ariel et al., 2009b; Chang-Liu and Woloschak, 1997; Wenger et al., 2004; Yu et al., 1997). The inconsistency in results between the BF2 cell lineages may be due to such mutations. The decline in ranavirus infectivity observed between 40 °C and 60 °C in the first experiment concerning cell susceptibility spurred investigations into exposure effect at two degree increments in this temperature range. As time of exposure and temperature increased, there was a decline in ranavirus infectivity resulting in total loss at 60 °C for 30 min and longer. The decline was observed to occur in two step-like phases, the first occurring between 40 and 44 °C and the second above 52 °C which may correspond to the loss of the viral envelope at the lower temperature around 40–44 °C and complete disruption of the envelope if not the whole virion above 52 °C. Previous studies have shown that there is a 10^3 drop of infectivity of the virus to cell cultures when the viral envelope is disrupted by solvents (Ariel et al., 1995). Also the envelope is needed for infectivity in cell culture but not *in vivo* (Aubertin, 1991). We speculate that the envelope is becoming disrupted but perhaps not fully disintegrating between 40 and 42 °C and is fully non-functional above 52 °C.

These results are very important in clarifying the paradigm that 32 °C is a critical temperature in ranavirus infection dynamics. It is clear that outside the animal, the virus is not even partially inactivated until temperatures are reached that are much higher (>40 °C) than 32 °C. Thus many of the higher temperature knockout effects reported in the literature (see Moody, 1992) could have been due to the cell's inability to replicate the virus at 32 °C as much as the virus itself being compromised. Careful assessment of previously published work needs to be undertaken. Therefore, the paradigm that the virus does not replicate above 32 °C could still be true, but this statement must not be understood to mean the virus is inactivated at 32 °C.

Although the immune system of ectothermic vertebrates is different to endothermic vertebrates, their ability to produce antibodies against pathogens is affected by environmental temperatures and many components of the non-specific and specific host response to infection are enhanced by small elevations in temperature (Bly and Clem, 1992; Coe, 1972; Zimmerman et al., 2010). Fever is part of the inflammatory response of vertebrates and although it is a physiological response (interaction between cytokines and the central nervous system) in endotherms (Conti et al., 2004), ectotherms such as reptiles must raise their body temperature behaviourally by basking or relocating to warmer areas of the environment. The upper limits of internal body temperatures of ranavirus hosts in the natural environment are unknown, though upper lethal body temperatures for many terrestrial reptiles are between 37 and 45 °C (Maness, 1979; Spellerberg, 1972). Although higher temperatures may result in recovery from infection, the results from this study suggest that if animals bask in warmer areas of their environment and increase their internal body temperatures; this 'behavioural fever' could reduce virus replication in infected hosts. Basking has previously been associated with diseased musk turtles (*Sternotherus depressus*), bog turtles (*Glyptemys muhlenbergii*), the common box turtle (*Terrapene carolina*) and the painted turtle (*Chyrsemys picta*) (Carter et al., 2005; Dodd, 1988; Monagas and Gatten, 1983). More importantly, studies of bacterial and viral-infected animals have suggested moderate fevers can decrease morbidity and increase survival rates. However, a host's susceptibility to infection is dependent on the interaction between the physiological activities of the pathogen and the defense responses of the host (Carey et al., 1999; Le Morvan et al., 1998). Consequently, this could affect host-pathogen interaction where there is a spatial or temporal variation in the thermal environment. The thermal environment may therefore be an important factor determining the distribution of ranaviruses in the environment.

This study defines the functional temperature range (<40 °C for little change in titre) that a ranavirus can tolerate outside the host by testing its' titer in cell culture under normal culture conditions after exposure to various temperatures and exposure times. The temperature tolerance of the virus in the environment is important when considering the transmission dynamics of the pathogen and disease. Indirect transmission is dependent on virion persistence in the environment and therefore the extended temperature tolerance of a BIV ranavirus as shown here impacts on the understanding of the epidemiology of the disease and has ramifications for biosecurity measures such as eradication, containments and control.

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References

Ariel, E., Jensen, B.B., 2009. Challenge studies of European stocks of redfin perch, *Perca fluviatilis L.*, and rainbow trout, *Oncorhynchus mykiss* (Walbaum), with epizootic haematopoietic necrosis virus. Journal of Fish Diseases 32, 1017–1025.

Ariel, E., Owens, L., Moody, N.J.G., 1995. A barramundi bioassay for iridovirus refractory to cell culture. In: Shariff, R.P., Subasinghe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture II. Fish Health Section. Asian Fisheries Society, Manila, Philippines, pp. 355–367.

Ariel, E., Kielgast, J., Svart, H.E., Larsen, K., Tapiovaara, H., Jensen, B.B., Holopainen, R., 2009a. Ranavirus in wild edible frogs (*Pelophylax* kl. *esculentus*) in Denmark. Diseases of Aquatic Organisms 85, 7–14.

Ariel, E., Nicolajsen, N., Christophersen, M.B., Holopainen, R., Tapiovaara, H., Jensen, B.B., 2009b. Propagation and isolation of ranavirus in cell culture. Aquaculture 294, 159–164.

Ariel, E., Holopainen, R., Olesen, N.J., Tapiovaara, H., 2010. Comparative study of ranavirus isolates from cod (*Gadus morhua*) and turbot (*Psetta maxima*) with reference to other ranaviruses. Archives of Virology 155, 261–271.

Aubertin, A.M., 1991. Iridoviridae. In: Francki, R.I.B., Fauquat, C.M., Knudson, D.L., Brown, F. (Eds.), Classification and Nomenclature of Viruses: Fifth Report of the International Committee on Taxonomy of Viruses, pp. 132–136.

Bly, J.E., Clem, L.W., 1992. Temperature and teleost immune functions. Fish & Shellfish Immunology 2, 159–171.

Carey, C., Cohen, N., Rollins Smith, L., 1999. Amphibian declines: an immunological perspective. Developmental and Comparative Immunology 23, 459–472.

Carter, S.L., Horne, B.D., Herman, D.W., Nichols, D.K., Haas, C.A., Mitchell, J.C., 2005. Bacterial pneumonia in free-ranging bog turtles, *Glyptemys muhlenbergii*, from North Carolina and Virginia. Journal of the North Carolina Academy of Science 121, 170–173.

Chang-Liu, C.M., Woloschak, G.E., 1997. Effect of passage number on cellular response to DNA-damaging agents: cell survival and gene expression. Cancer Letters 113, 77–86.

Chinchar, V.G., Hyatt, A., Miyazaki, T., Williams, T., 2009. Family Iridoviridae: Poor viral relations no longer. In: van Etten, J. (Ed.), Lesser Known Large dsDNA Viruses. Springer, Berlin, pp. 123–170.

Coe, J.E., 1972. Immune response in the turtle (*Chrysemys picta*). Immunology 23, 45–52.

Conti, B., Tabarean, I., Andrei, C., Bartfai, T., 2004. Cytokines and fever. Frontiers in Bioscience 9, 1433–1449.
Crane, M.S.J., Young, J., Williams, L.M., 2005. Epizootic haematopoietic necrosis virus (EHNV): Growth in fish cell lines at different temperatures. Bulletin of the European Association of Fish Pathologists 25, 228–231.
De Voe, R., Geissler, K., Elmore, S., Rotstein, D., Lewbart, G., Guy, J., 2004. Ranavirus associated morbidity and mortality in a group of captive eastern box turtles (*Terrapene carolina carolina*). Journal of Zoo and Wildlife Medicine 35, 534–543.
Dodd, C.K., 1988. Disease and population declines in the flattened musk turtle *Sternotherus depressus*. American Midland Naturalist 119, 394–401.
Fijan, N., Sulimanovic, D., Bearzotti, M., Muzinic, D., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J.F., de Kinkelin, P., 1983. Some proterties of the Epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio*. Annales de l'Institut Pasteur. Virology 134, 207–220.
Granoff, A., Came, P.E., Rafferty, K.A., 1965. The isolation and properties of viruses from *Rana pipiens*: their possible relationship to the renal adenocarcinoma of the leopard frog. Annals of the New York Academy of Sciences 126, 237–255.
Gravell, M., Granoff, A., 1970. Viruses and renal carcinoma of Rana pipiens* 1: IX. The influence of temperature and host cell on replication of frog polyhedral cytoplasmic deoxyribovirus (PCDV). Virology 41, 596–602.
Johnson, A.J., Pessier, A.P., Wellehan, J.F.X., Childress, A., Norton, T.M., Stedman, N.L., Bloom, D.C., Belzer, W., Titus, V.R., Wagner, R., Brooks, J.W., Spratt, J., Jacobson, E.R., 2008. Ranavirus infection of free-ranging and captive box turtles and tortoises in the United States. Journal of Wildlife Diseases 44, 851–863.
Langdon, J., Humphrey, J., Williams, L.M., 1988. Outbreaks of an EHNV like iridovirus in cultured rainbow trout, *Salmo gairdneri* Richardson, in Australia. Journal of Fish Diseases 11, 93–96.
Le Morvan, C., Troutaud, D., Deschaux, P., 1998. Differential effects of temperature on specific and nonspecific immune defences in fish. Journal of Experimental Biology 201, 165–168.
Maness, J.D., 1979. The role of behaviour in temperature acclimation and tolerance in ectotherms. American Zoologist 19, 367–384.
Monagas, W.R., Gatten, R.E., 1983. Behavioural fever in the turtles *Terrapene carolina* and *Chrysemys picta*. Journal of Thermal Biology 8, 285–288.
Moody, N.J.G., 1992. Experimental infection of barramundi *Lates calcarifer* with Bohle iridovirus. BSc Hons Department of Biomedical and Tropical Veterinary Sciences, James Cook University of North Queensland. 106 pp.
OIE, 2008. Aquatic Animal Health Code. World Organisation for Animal Health, Paris, France.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology 27, 493.
Rojas, S.K., Richards, K., Jancovich, J.K., Davidson, E.W., 2005. Influence of temperature on Ranavirus infection in larval salamanders *Ambystoma tigrinum*. Diseases of Aquatic Organisms 63, 95–100.
Speare, R., Smith, J., 1992. An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. Diseases of Aquatic Organisms 14, 51–57.
Spellerberg, I.F., 1972. Temperature tolerances of southeast Australian reptiles examined in relation to reptile thermoregulatory behaviour and distribution. Oecologia 9, 23–46.
Wenger, S.L., Senft, J.R., Sargent, L.M., Bamezai, R., Bairwa, N., Grant, S.G., 2004. Comparison of established cell lines at different passages by karyotype and comparative genomic hybridization. Bioscience Reports 24, 631–639.
Whittington, R., Reddacliff, G., 1995. Influence of environmental temperature on experimental infection of redfin perch (*Perca fluviatilis*) and rainbow trout (*Oncorhynchus mykiss*) with epizootic haematopoietic necrosis virus, an Australian iridovirus. Australian Veterinary Journal 72, 421–424.
Wolf, K., Gravell, M., Malsberger, R.G., 1966. Lymphocystis virus: isolation and propagation in centrarchid fish cell lines. Science 151, 1004.
Wright, R.K., Cooper, E.L., 1981. Temperature effects of ectotherm immune responses. Developmental and Comparative Immunology 5, 117–122.
Yu, H., Cook, T.J., Sinko, P.J., 1997. Evidence for dimished functional expression of intestinal transporters in Caco-2 cell monolayers at high passages. Pharmaceutical Research 14, 757–762.
Zimmerman, L.M., Vogel, L.A., Bowden, R.M., 2010. Understanding the vertebrate immune system: insights from the reptillian perspective. Journal of Experimental Biology 213, 661–671.