Stability of infectivity of systemic iridoviruses during storage at +4°C and -70°C

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Introduction

Experimental challenge experiments are currently underway to assess the susceptibility of selected fish and amphibian species to a panel of systemic iridoviruses. This work forms part of the European Union FP6 project "RANA" (Risk assessment of new and emerging systemic iridoviral diseases for European fish and aquatic ecosystems). Prior to the commencement of challenge experiments, it was necessary to determine the stability of infectivity of the virus isolate panel. This was so that all animals could be challenged with a standardised viral titre. Stability of infectivity was measured during storage at +4°C and -70°C. A temperature of +4°C was chosen to establish if virus could be stored in the refrigerator for short periods after titration (i.e. prior to use in an experimental challenge) without loss of titre. A temperature of -70°C was chosen to see if long-term storage of virus was possible at -70°C, without significant loss of titre. Stability of infectivity was measured by calculating $TCID_{50}$ values for each virus isolate, after propagation in bluegill fry (BF-2) cell culture, prior to and after storage for 3, 6 and 12 months.

Materials and methods

Virus isolates

Table 1 lists the 9 virus isolates used in the study, their host and country of isolation. All viruses were characterised and distributed by the European Community Reference Laboratory for Fish Diseases, Aarhus, Denmark.

Table 1: Systemic iridoviruses for which stability of infectivity was tested, their host and country of isolation.

Virus	Host	Country of isolation
Epizootic haematopoietic necrosis virus (EHNV)	Redfin perch, *Perca fluviatilis*	Australia
European catfish virus (ECV)	Catfish, *Ictalurus melas*	Italy
European sheatfish virus (ESV)	Sheatfish, *Siluris glanis*	Germany
Doctor fish virus (DFV)	Doctor fish, *Labroides dimidatus*	USA
Guppy virus 6 (GV6)	Guppy, *Poecilia reticulata*	USA
Pike-perch iridovirus (PPIV)	Pike-perch, *Stizostedion lucioperca*	Finland
New Zealand eel iridovirus (NZ eel IV)	Eel, *Anguilla australis*	Italy
Bohle iridovirus (BIV)	Ornate burrowing frog, *Limnodynastes ornatus*	Australia
Rana esculenta virus – like iridovirus (REV-like)	Green frog, *Rana esculenta*	Italy

Stability experiments

On receipt at the laboratory, virus was cultivated in BF-2 cell culture in 175cm^2 flasks with Glasgow minimum essential medium (GMEM) + 2% foetal bovine serum (FBS) + antibiotic solution (all Sigma Aldrich Ltd. UK). Flasks were incubated at a temperature consistent with published information for each virus. Flasks were observed daily and removed to +4°C once 100% cytopathic effect (CPE) was reached. For all viruses 100% CPE occurred after 1-5 days incubation. Virus suspension was held at +4°C for no longer than 24 hours, after which it was aliquoted, in 600µl volumes, into sterile cryovials and stored at +4°C or -70°C. Prior to storage (Time 0) a volume of virus suspension was titrated. Virus was serially diluted to a 10^{-9} dilution of the original suspension (using 2% FBS GMEM + antibiotics) and 10^{-5} to10^{-9} dilutions added to 96 well trays of BF-2 cells. 8 wells were inoculated per dilution. Trays were incubated in a moist chamber at the same temperature virus was initially grown, and cells examined periodically for the presence of CPE. After 7 days incubation, $TCID_{50}$ values were calculated for each virus using the method of Reed and Muench (1938). After periods of 3, 6 and 12 months, cryovials, containing 600µl aliquots of each virus, were removed from storage at +4°C and -70°C, and allowed to reach room temperature by leaving on the bench for no longer than 2 hours. Virus was then re-titrated using exactly the same methods as described above.

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Results

Table 2 shows the infectivity of the 9 viruses after storage for 3, 6 and 12 months at +4°C and -80°C. Figure 1 shows the infectivity of each virus after 12 months storage at each temperature. The infectivity of all the viruses tested was not significantly reduced by storage at +4°C or -70°C for periods of at least 12 months.

Figure 1: Infectivity of 9 systemic iridoviruses after storage at +4°C and –70°C for one year. Virus titres were determined by calculating $TCID_{50}$ values for each isolate after propagation in BF-2 cell culture, prior to (T0) and after storage, and are expressed as log_{10} per ml.

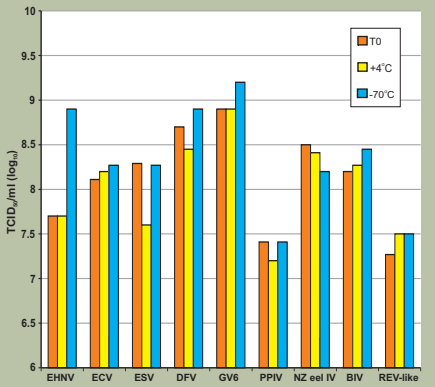

Table 2: Infectivity of 9 systemic iridoviruses after storage at +4°C and -70°C in culture media for 3, 6 and 12 months. Virus titres were determined by calculating $TCID_{50}$ values for each virus isolate, after propagation in BF-2 cell culture, prior to and after storage, and are expressed as log_{10} per ml.

Virus	Storage temperature (°C)	Storage time			
		Time 0	3 months	6 months	12 months
EHNV	+4	7.70	7.9	8.03	7.70
	-70	7.70	9.13	9.20	8.90
ECV	+4	8.11	7.6	7.90	8.20
	-70	8.11	8.2	8.47	8.27
ESV	+4	8.29	7.5	7.50	7.60
	-70	8.29	8.37	8.99	8.27
DFV	+4	8.70	9.20	9.27	8.45
	-70	8.70	8.93	8.99	8.90
GV6	+4	8.90	9.27	8.70	8.90
	-70	8.90	8.60	9.13	9.20
PPIV	+4	7.41	7.37	7.13	7.20
	-70	7.41	7.37	7.29	7.41
NZ eel IV	+4	8.50	8.37	8.60	8.41
	-70	8.50	8.41	8.45	8.20
BIV	+4	8.20	8.34	8.03	8.27
	-70	8.20	8.99	9.27	8.45
REV-like	+4	7.27	7.45	8.13	7.50
	-70	7.27	8.90	8.20	7.50

Conclusions

- Infectivity of the nine systemic iridoviruses tested is stable during storage at +4°C and -70°C for at least a year. Virus can therefore be stored for periods of at least 12 months at -70°C in between experimental challenge experiments. After propagation and titration for a challenge experiment, virus could also be stored at +4°C prior to use, with insignificant loss of titre.
- The freeze-thaw process does not appear to significantly decrease the infectivity of the viruses. This means that during challenge experiments, mortalities could be frozen prior to virus re-isolation tests, if there is insufficient time to process animals immediately. Further work is however necessary to ensure virus is as stable in animal tissue as when stored in culture media since the protein enriched culture medium could have a beneficial effect on virus survival. There is little published information on the stability of infectivity of systemic iridoviruses, however Langdon (1989) reported that infectivity was still present in EHNV-infected fish tissue and culture medium stored at -20°C and -70°C for two years.
- For some viruses tested, particularly EHNV, infectivity appeared to increase after virus had been frozen. A possible reason for this is that the freeze-thaw process may cause any clumps of virus to disaggregate, and thereby release more individual viral particles.
- The stability of infectivity of the systemic iridoviruses tested compares favourably to that of some other fish viruses. For instance, Burke and Mulcahy (1983) demonstrated a 0.5 log_{10} reduction in infectivity for infectious haematopoietic necrosis virus after 5 weeks storage at +4°C in Eagle's MEM with Earle's salts. When the virus (1.1 X 10^4 plaque forming units per ml) was stored at -20°C, there was no infectious virus detectable after 2 weeks storage. Infectious pancreatic necrosis virus showed no significant change in titre after storage at +4°C for 7 weeks in Eagle's MEM with 2.5% foetal calf serum, however after this time loss of titre was exponential throughout the 9 month test period. An approximate loss in titre of 0.5 log_{10} was demonstrated after storage at -70°C for the same period (Malsberger and Cerini, 1963).