Cryobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Protocol for cryopreservation of the turbot parasite *Philasterides dicentrarchi (Ciliophora, Scuticociliatia)*

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ARTICLE INFO

Keywords:
Philasterides dicentrarchi
Miamiensis avidus
Freezing
Cryoprotectants
Cryopreservation
Optimization

ABSTRACT

Philasterides dicentrarchi is a free-living marine ciliate that can become an endoparasite that causes a severe disease called scuticociliatosis in cultured fish. Long-term maintenance of this scuticociliate in the laboratory is currently only possible by subculture, with periodic passage in fish to maintain the virulence of the isolates. In this study, we developed and optimized a cryopreservation protocol similar to that used for the long-term storage of scuticociliates of the genus *Miamiensis*. The cryogenic medium comprised ATCC medium 1651 and a combination of 11% dimethylsulfoxide and 5% glycerol. We have verified that the most important factor ensuring the efficiency of the cryopreservation procedure is the growth phase of the culture, and that ciliates should be cryopreserved at the stationary phase (around the sixth day of culture). The cryopreservation protocol described here can be used for all strains of *P. dicentrarchi* as well as commercial strains of *Miamiensis* and enables the virulence of the strains to be maintained. Finally, this cryopreservation protocol has been shown to be more effective than others routinely applied to scuticociliates, yielding a higher survival rate with a lower initial concentration of ciliates. The results obtained indicate that the cropreservation protocol enables the long-term storage of scuticociliate parasites while maintaining the virulence of the isolates. The protocol is therefore suitable for use in vaccine production and related studies.

1. Introduction

Scuticociliates (*Ciliophora*: *Scuticociliatia*) are ubiquitous members of phagotrophic marine free-living ciliates that colonize the marine environment and are abundant in coastal waters [30]. Some species can also act as opportunistic histophagous parasites, causing systemic infections in marine cultured fish worldwide [26]. Such infections usually lead to rapid death of the host [15,36]. Scuticociliatosis, which is caused by *Philaterides dicentarchi* and affects cultured flatfish such as the turbot *Scophthalmus maximus*, the olive flounder *Paralichthys olivaceus* and the fine flounder *Paralichthys adspersus*, is particularly serious and causes high mortalities in infected fish [9,18,21].

Techniques, used to maintain protozoan species and strains in the laboratory for long periods of time without frequent subculture, include drying, freeze-drying, and in particular, cryopreservation [20,24].

Cryopreservation at ultra-low temperatures has been applied to several species of parasites, thus eliminating the need to maintain cultures by repeated passage *in vitro*. Cryopreservation is the method of choice for long-term maintenance as it overcomes the disadvantages associated with *in vitro* propagation, e.g. the length of the procedures used, difficulty in initial isolation, loss of strains, bacterial and fungal contamination during handling, changes in the original biological and metabolic characteristics, and the re-establishment of infections *in vivo* [10,20,24]. Serial subculture, particularly of axenic strains, can also result in the loss of biological characteristics such as virulence [22].

Although successful cryopreservation of some non-encysting ciliates is quite easy to achieve, the procedure is difficult for other ciliates, including the scuticociliates, and modifications must be made to the normal thawing procedure [28]. Strains of the marine scuticociliates belonging to the genera *Methanophrys, Miamiensis, Parauronema, Paranophrys, Pseudocohnilembus* and *Uronema* can be maintained in an axenic state [34] and cryopreserved in formulations containing glycerol, methanol and dimethyl sulphoxide (Me_2SO) as cryoprotective agents [2,33]. *P. dicentrachi* can also be maintained in axenic cultures in Leibovitz L15 medium supplemented with foetal bovine serum, lipids, nucleosides and glucose [17]. The ciliates obtained by this method retain their capacity to infect turbot after at least twenty passages *in vitro*. However, the virulence of the isolates is modified by long-term *in vitro* culture [1,35], and isolates must be passaged in turbot to recover their virulence [31]. Several studies have demonstrated the existence of

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https://doi.org/10.1016/j.cryobiol.2017.11.010
Received 13 July 2017; Received in revised form 20 November 2017; Accepted 21 November 2017

an intraspecific variation in *P. dicentrarchi* that displays clear antigenic differences between strains and which can be altered during serial subculture, thus affecting the protection afforded by vaccination [6,7,32]. Cryopreservation would overcome the disadvantages associated with serial culture (also observed in other protozoan parasites), such as the labour-intensive techniques involved, bacterial/fungal contamination of cultures, genetic drift, and loss of the infectivity and immunogenicity of the strains [5,24,36].

Cryopreservation of the scuticociliate parasite *P. dicentrarchi* has not yet previously been reported. The objective of this study was to develop and optimize an effective cryopreservation protocol for the long-term storage of the turbot parasite *Philasterides dicentrarchi*.

2. Materials and methods

2.1. Scuticociliate isolation and culture

Isolates of the scuticociliate *P. dicentrarchi* were obtained from the ascitic fluid of naturally infected turbot during an outbreak of scuticociliatosis affecting different turbot farms in Galicia (NW Spain) and Portugal [6]. The farms were located in the Ria de Muros-Noia (isolate S1), the Ria de Arousa (isolates I1, C1, D2, D3; isolates D2 and D3 were obtained from the same fish farm), the Ria de Vigo (isolate B1) and the Algarve (southern Portugal; isolate Po). Two isolates of *P. dicentrarchi*, denominated Pe5 and Pe7, were also obtained from the ascitic fluid of naturally infected specimens of the fine flounder *Paralichthys adspersus* from a fish farm in Peru (Ancash, Huarmey Province) [9], as previously described [18]. The *P. dicentrarchi* isolates were maintained in the laboratory in Leibovitz L15 medium supplemented with 10% foetal bovine serum, lipids (lecithin and Tween 80), nucleosides and glucose (standard complete L15 medium), under the culture conditions described by Iglesias et al. [17].

Strains Ma and Ma/2 of *Miamiensis avidus* deposited by A.T. Soldo and E. B. Small under the name *Miamiensis avidus* Thompson and Moewus (ATCC® 50179™ and ATCC® 50180™, respectively) were acquired from the American Type Culture Collection (ATCC, USA). Strains Ma and Ma/2 of *M. avidus* were cultivated axenically in ATCC® medium 1651 MA (LGC Standards, Spain) at 25 °C and were subcultured every 3–5 days.

2.2. Freezing protocol for cryopreservation

The ciliates were collected from cultures at different stages of growth and centrifuged at 700 × g for 5 min. Aliquots of 0.5×10^6 ciliates were resuspended in 0.5 mL of 1651 MA medium diluted 1:1 in double distilled water (MM), in Leibovittz L15 medium, or in artificial sea water (in g/L solution: 23.926 g NaCl, 4.008 g Na_2SO_4, 0.677 g KCl, 0.196 g $NaHCO_3$, 0.098 g KBr, 0.026 g H_3BO_3, 10 g Cl_2Mg, 2.22 g $CaCl_2$) (ASW), plus 5, 7.5 or 10% glycerol or 11% Me_2SO, in 1.8 mL sterile plastic screw-top cryotubes (Nunc, Thermo Scientific, China). Cell preparation took between 5 and 10 min and never exceeded 15 min. Finally, the cryotubes were placed in a cell freezing container (CoolCell, Biocision), which decreased the temperature by approximately 1 °C/min, before being transferred to a freezer (−80 °C) for 2 h and then stored in liquid nitrogen. In some experiments, the same medium with cryoprotectant additives (CPA) was used, but with the glycerol replaced by 5% methanol.

2.3. Thawing steps

For thawing, the cryovials were removed from the liquid nitrogen and immediately placed in a water bath at 37° C for 2 min, without shaking, ensuring that the frozen samples were completely submerged. The cryoprotective solution was then removed by centrifugation at 750*g* for 5 min at room temperature. The cell pellet was resuspended in 0.5 mL of MM, under aseptic conditions, and the mixture was transferred to a sterile 15 mL Falcon ™ conical bottom tube with an additional 2.0 mL of MM medium. Two cryovials were used in each experiment, and the contents (total 5.0 mL) were aseptically transferred to a 25 cm^2 vented flask (Corning™ Cell Culture Treated Flasks). The flasks were incubated horizontally in a cooled incubator at 21 °C, and cell growth was monitored daily by examination under an inverted microscope.

2.4. Viability/survival assessment and in vitro growth

Cultures were examined on an inverted microscope (Eclipse CFL60, Nikon, Japan) at 200x magnification to check viability and ciliate morphology. The viability/survival of *P. dicentrarchi* trophozoites was assessed by motility. The number of motile ciliates present in different parts of the flask were counted. Results were expressed as the number of motile ciliates (NMC) per flak, calculated using the following formula:

Survival level following cryopreservation = NMC/flask = (NMC1 x S / Sf x Nf) x V,

where,

NMC1: Number of motile ciliates present in the sample after thawing
S: flask surface (25×10^2 mm^2)
Sf: Surface area of a visual field in the 20X objective lens (0.95 mm^2)
Nf: Number of fields displayed (n = 5)
V: Total sample volume (5 mL)

Three flasks were examined at each time point and the mean values (and standard deviations) for recovery quantified at 24, 48 and 72 h after thawing were calculated, and expressed as a percentage (%) of the total number of ciliates for each experiment.

To determine the *in vitro* growth of the different strains of *P. dicentrarchi*, 75 cm^2 flasks were inoculated with 5×10^4 ciliates/mL in a final volume of 30 mL of MM medium. Aliquots (15 μL) of medium were removed daily from each flask and ciliates inactivated by addition of 0.25% glutaraldehyde (Sigma-Aldrich) before being quantified using a haemocytometer [17].

2.5. Experimental design

We investigated the effects of two controllable components of cryopreservation on the viability of *P. dicentrarchi*: the concentration of the cryoprotectant glycerol (factor A) and the day of culture (factor B). Experiments were designed using STATGRAPHICS Centurion XVI version 16.1.15 Windows (Statgraphics.net, Madrid) by application of a 3^2 factorial design, as previously described [23]. StatAdvisor created a factorial design with 10 experimental runs to study the effects of each factor on the viability of samples 24 h after thawing (Table 2). Experiments were executed in a single block with the order randomized to limit the effect of hidden variables.

For experimental infections, two groups of fifteen fish were infected intraperitoneally with 0.1 mL of 10^6 ciliates/ml in phosphate-buffered saline (PBS) containing 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, and 137 mM NaCl, as previously described [31]. The fish were held according to the criteria of protection, control, care and welfare of animals and the legislative requirements relating to the use of animals