Received: 14 July 2020 | Revised: 13 October 2020 | Accepted: 14 October 2020

DOI: 10.1111/jfd.13295

ORIGINAL ARTICLE

Journal of Fish Diseases WILEY

Method for cryopreservation of *Paramoeba perurans*

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Funding information
This work has been funded by the Norwegian Seafood Research Fund (FHF, Fiskeri- og havbruksnæringens forskningsfinansiering), project 901053.

Abstract

Paramoeba perurans causes amoebic gill disease (AGD), which is a major problem in aquaculture worldwide. The parasite can be cultured *in vitro*, but to this date, no method for long-term storage of the clones exists. In this study, we describe a method for cryopreservation of *Paramoeba perurans*. The method was successfully employed on four out the five clones we tested. The thawing success rate, that is the percentage of successfully thawed vials relative to the total number of vials that were thawed, differed for the clones and ranged from 25% to 100%. The age of the clones seemed to have a negative impact on the ability to survive cryopreservation.

KEYWORDS
cryopreservation, *Neoparamoeba*, *Paramoeba*, *perurans*

1 | BACKGROUND

Gill diseases constitute a major problem in Norwegian salmon (*Salmo salar* L.) farming, and in 2006, the industry experienced the first outbreaks of amoebic gill disease (AGD) caused by a new species of *Paramoeba* (Nylund et al., 2007, 2008; Steinum et al., 2008). The amoeba was later characterized, named *Paramoeba perurans* (Syn. *Neoparamoeaba perurans*), and shown to be the causative agent of AGD also in Australia (Crosbie et al., 2012; Young et al., 2007). This parasite has become a serious problem for aquaculture industry worldwide (Bustos et al., 2011; Downes et al., 2015; Dykova et al. 2000b; Karlsbakk et al., 2013; Kim et al., 2005, 2016; Mouton et al., 2014; Munday et al., 2001; Nowak et al., 2002; Nylund et al., 2007, 2008; Oldham et al., 2016; Rodger, 2014; Steinum et al., 2008; Young et al., 2008). To gain a better understanding of the disease and *P. perurans*, controlled challenge experiments have been performed with field isolates or cultured clones derived from field isolates (Collins et al., 2017; Crosbie et al., 2010, 2012; Dahle et al., 2020; Haugland et al., 2017; Kindt, 2017; Røed, 2016; Taylor et al., 2009), and the *P. perurans* clones have been shown to display variation in virulence (Collins et al., 2017; Crosbie et al., 2010; Dahle et al., 2020; Kindt, 2017; Røed, 2016). The use of cloned cultures in challenge experiments is generally thought to increase the reproducibility of such experiments. However, there have been reports of loss of virulence for clones/isolates that have been kept over longer periods (Bridle et al., 2015). In addition, keeping an isolate or a clone of *P. perurans* requires frequent attention and passages to new media every two or three weeks which is quite time consuming. Thus, a procedure for cryopreservation of *P. perurans*, reducing the workload and securing clones of *P. perurans* that give reproducible result during challenge studies, is needed. Since we started working with *P. perurans* in 2013, we have been focusing on finding a procedure for cryopreservation for our cloned isolates of *P. perurans*. Cryopreservation has been used for other amoebae (Dykova et al. 2000a; Kalinina & Page, 1992; Seo et al., 1992), and we found, using existing methods, that it also worked for *Paramoeba pemaquidensis* isolated from fish species in Norway. Starting with existing cryopreservation protocols, we have developed a method for cryopreservation of *P. perurans*.

2 | MATERIALS AND METHODS

2.1 | Maintenance culture

Xenic clonal cultures have been established from primary field isolates by propagation of single cells that had been separated from a cell suspension with a pipette. The clones were cultured at 16°C in T75 cell culture flasks with liquid medium, as described by Haugland et al. (2017). The medium was malt yeast broth (MYB): 0.1% w/v malt extract and 0.1% w/v yeast extract dissolved in sea water (34 practical salinity units) and autoclaved. The cultures were kept in the dark, and every second week, they were passaged by pouring the culture supernatant containing floating amoebae into a new flask. When sufficient numbers of amoebae had attached to the bottom of the new flask (normally after 30 min and up to two hours), the old medium was poured out and replaced with fresh MYB.

A total of five clonal cultures of *P. perurans* collected from different locations in Norway over a period of 2.5 years were included in this study (Table 1). Notably, one of the clones, H02/13Pp, was not associated with the intracellular bacterium *Candidatus* Syngnamydia salmonis, Chlamydiales (Nylund et al., 2015, 2018), as assessed by qRT-PCR (Nylund et al., 2015).

2.2 | Freezing of amoebae

The protocols for freezing and thawing of *P. perurans* were inspired by cryopreservation protocols developed and optimized for other amoeba (Kalinina & Page, 1992; Seo et al., 1992; Holzer, pers. comm.; ATCC® Culture method for *P. pemaquidensis*: https://www.lgcstandards-atcc.org/products/all/30735.aspx#culturemethod; ATCC® Protistology Culture Guide: https://www.atcc.org/~/media/PDFs/Culture%20Guides/ProtistologyGuide.ashx).

TABLE 1 All clones of *P. perurans* were obtained from gills of Atlantic salmon (*Salmo salar* L.). The code of the clones include: 1) the region the isolate was taken from (the first letter(s)), 2) followed by the isolate number from that region, 3) the year of isolation and 4) Pp for *Paramoeba perurans*

Code	Region	Date	Passages in culture
H02/13Pp	Hordaland (West Norway)	October 2013	94
H03/14Pp	Hordaland (West Norway)	22.01.2014	98
R18/15Pp	Rogaland (Southwest Norway)	04.09.2015	61
ST19/15Pp	Sør-Trøndelag (Mid Norway)	13.10.2015	59
H20/16Pp	Hordaland (West Norway)	05.02.2016	53

To stimulate growth and accumulate enough cells, the passage frequency was increased to every second or third day for 2–3 weeks. During this time, all flasks were continued instead of disposing the old passages. All cells were then harvested—floating cells by pouring out the medium and adherent cells by using a cell scraper and rinsing out the flasks with sterile-filtered autoclaved sea water. The amoebae were concentrated by centrifugation at 1,500 g for ten minutes, the medium removed, and the cells resuspended in a small volume of sterile-filtered autoclaved sea water. The same volume of freezing medium (20% dimethyl sulfoxide (DMSO) in sterile-filtered autoclaved sea water) was added, and the cell suspension mixed carefully, leading to a final concentration of 10% DMSO. Approximately $2–6*10^5$ cells in aliquots of 300–800 µl were added to cryogenic vials, which were placed in a Nalgene Mr. Frosty freezing container filled with 100% isopropyl alcohol to facilitate slow freezing. After about 20 min at room temperature, the cells were frozen slowly at a controlled rate (1°C/min) by placing the container at −80°C for two to three hours before storing the tubes in liquid nitrogen.

A total of twelve freezing experiments with at least two batches from each of the five cloned isolates were performed (Table 2). In four of these tests, adherent cells and floating cells were collected in different tubes and frozen separately to determine whether pseudocyst formation (Lima et al., 2017) is advantageous for cryopreservation of *P. perurans*.

The total number of *P. perurans*, before cryopreservation, was determined in a CASY Model TT Cell counter (Innovatis, Roche Diagnostics) for four batches, and the number of amoebae in each frozen ampoule calculated. For the other eight freezing experiments, visual inspection of amoebae density in the culture flasks before cryopreservation with a microscope confirmed amoebae numbers in the same range.

2.3 | Thawing of amoebae

After rapid thawing in a water bath at about 35 °C for roughly 30 s, the cells were incubated at 16 °C in T75 cell culture flasks containing MYB for four weeks or until proliferating attached amoebae were observed (Table 2). Cell viability was not tested after thawing, and it was not possible to distinguish live from dead amoebae among the floating cells. When attached amoebae were observed for the first time in a flask, it was usually only between one and five cells. These surviving cells would then start to proliferate forming small islands and eventually spread over the whole surface of the flask, behaving seemingly like under maintenance culture conditions. The recovery rate for each vial is defined as the percentage of surviving cells relative to the total number of frozen cells. In the absence of a precise number for surviving cells, we used the conservative estimate of one surviving cell per vial in the calculation. The term "successful" thawing is used in this paper if at least one cell per vial survived. The "success rate" denotes for each clone the percentage of successfully thawed vials relative to the total number of vials that were thawed.

To remove DMSO, due to its potential for cytotoxicity, cells were initially either centrifuged for five minutes at 1,000 g directly after thawing and the supernatant removed before transfer to cell culture flasks, or the medium in the flasks exchanged after two hours of incubation. However, a simple test did not show any negative effects of DMSO on amoeba growth when compared to normal culture conditions. In short, three *P. perurans* clones from maintenance cultures were either exposed to DMSO (in the concentration expected in the culture medium after diluting one tube of frozen amoebae in 20 ml MYB) or cultured in MYB for 16 days at 16℃. The experiment was carried out in triplicate wells for each clone on 6-well plates. Manual visual inspection of amoeba density in a microscope did neither reveal differences between the technical parallels nor between wells with and without DMSO. Thus, in the later thawing attempts the freezing medium was not removed from the cell suspension.

The effect of freezing and thawing on the composition of the bacterial community in the amoeba cultures has not been studied. It can be assumed that bacteria take less damage than amoebae during the process, but different bacteria could be affected differently. Changes in the composition of the bacterial community in the culture medium might in turn affect the amoebae negatively. Therefore, the defrosted amoebae were cultured in "conditioned medium" in several thawing tests. "Conditioned medium" was made of MYB from maintenance cultures of the respective cloned isolates by filtration through a 1.2-μm syringe filter to remove all amoebae, but leave the bacteria present in those cultures. The absence of living amoebae in "conditioned medium" was confirmed by incubation in T75 cell culture flasks at 16 ℃ over several weeks where no growth was observed. With the use of "conditioned medium," we aimed to provide the freshly thawed amoebae optimal nutrition in the form of the live bacterial community they grew in before cryopreservation.

3 | RESULTS AND DISCUSSION

Five cloned isolates were used in this study. At least two batches of each clone had been frozen, resulting in a total of twelve separate freezing tests. Between one and six vials from the same frozen batch were thawed. Twenty-nine thawing attempts were conducted in total. Table 2 shows detailed information for all cryopreservation experiments deployed in this study. An overview of the number of thawed vials for each clone and the thawing success rate is given in Figure 1. Thirteen vials were thawed successfully, that is at least one cell had survived cryopreservation in the respective vials and started to proliferate seemingly normally under standard culture conditions in our laboratory.

One of the cloned isolates (H20/16Pp) was thawed with a 100% success rate (i.e. seven out of seven vials, originating from two different frozen batches, were thawed successfully). The success rate was 75% for ST19/15Pp (three out of four vials; from two different frozen batches), 50% for R18/15Pp (two out of four vials; from two different frozen batches) and 25% for H03/14Pp (one out of four vials; from two different frozen batches). H02/13Pp could not be successfully thawed (none out of ten vials; from four different frozen batches). These results suggest that the age of the culture could have a negative effect on its potential for cryopreservation. Also, the absence of the intracellular bacterium *Candidatus* Syngnamydia salmonis (Nylund et al., 2015, 2018) in H02/13Pp could be a contributing factor.

For the four batches for which amoebae were counted before freezing, there were on average $2.2*10^5$, $3.6*10^5$, $5.6*10^5$ and $5.4*10^5$ amoebae per vial, respectively. Similar numbers were determined for the other eight batches. This corresponded well with the recommended number of cells for freezing *P. pemaquidensis*, which is $5*10^5$ cells per cryovial (ATCC® Culture method for *P. pemaquidensis*: https://www.lgcstandards-atcc.org/products/all/30735.aspx#culturemethod), as well as other amoebae (Kalinina & Page, 1992). In our tests, few cells from each vial survived the cryopreservation process. Assuming that only one cell survived, a conservative estimate of the cell recovery rate (percentage of cells surviving the freeze and thaw process) would be between 0.0002% and 0.0005%. This is several orders of magnitude below the standard recovery rate of 1% suggested by Kalinina and Page (1992).

The first attached and proliferating amoebae were observed in the cell culture flasks between seven and 23 days after thawing. This means that none of the surviving cells were able to spontaneously attach to the flasks like they would after a normal passage. To investigate whether pseudocysts of *P. perurans* (Lima et al., 2017) were more resilient to the cryopreservation process, we froze floating and attached cells from the same culture flasks separately for four batches. However, only one of these batches could be thawed successfully. Based on the results from this batch, we could not determine a difference in survival between floating and attached cells. This question should be examined in more depth in future experiments.

Amoebae were thawed between one day and 1.5 years after freezing. The duration of the time that clones of *P. perurans* were frozen did not seem to influence the thawing success rate for the time span examined in this study.

Out of the 13 thawing experiments where the use of "conditioned medium" was compared to MYB, five were successful. "Conditioned medium" was beneficial compared to fresh MYB in two out of these five tests and performed equally with MYB in one. The disadvantage of using "conditioned medium" is that the amount of nutrients and metabolic waste products in the media is not known. Adding a specific amount of a well-characterized clone of live or inactivated bacteria to fresh medium would overcome this problem. More research is needed to determine whether the addition of bacteria to the freshly thawed amoebae is advantageous for their survival or whether the bacteria present in the culture and frozen together with the amoebae provide enough nutrition.

TABLE 2 Detailed overview of the cryopreservation experiments deployed in this study. In total, twelve batches of amoeba were frozen, and 29 vials thawed. "Conditioned medium" was abbreviated cond. MYB

Clone	H20/16Pp	R18/15Pp	ST19/15Pp	ST19/15Pp	H02/13Pp
Date frozen	2016–10–25	2016–11–22	2016–11–30	2016–11–30	2016–12–07
Last passage	2016–10–18	2016–10–18	2016–10–18	2016–10–18	2016–11–22
Days after last passage	7	35	43	43	15
Attached and floating amoebae frozen separately	Yes	No	No	No	Yes
n amoebae before freezing	2,374,566	2,851,275	4,458,538	4,344,175	N/A
n vials	11	8	8	8	9
µl per vial	6*800 floating, 5*800 attached	600	600	600	6*800 floating, 3*300 attached
n amoebae per vial	215,870	356,409	557,317	543,022	N/A
1st vial thawed	31/10/2016	01/12/2016	01/12/2016	01/12/2016	06/01/2017
State before freezing	Attached	Attached + floating	Attached + floating	Attached + floating	Attached
Days after freezing	6	9	1	1	30
Days until first living amoebae observed	14	17	15	N/A	N/A
2nd vial thawed	2016–10–31	2017–01–06	2017–01–06	2017–01–06	2017–01–06
State before freezing	Floating	Attached + floating	Attached + floating	Attached + floating	Floating
Days after freezing	6	45	37	37	30
Days until first living amoebae observed	14	N/A	12	12	N/A
3rd vial thawed	2017–01–06				2017–11–30
State before freezing	Attached				Attached
Days after freezing	73				358
Days until first living amoebae observed	7				N/A
4th vial thawed	2017–01–06				2017–11–30
State before freezing	Floating				Floating
Days after freezing	73				358
Days until first living amoebae observed	7				N/A
5th vial thawed	2017–11–30				
State before freezing	Floating				
Days after freezing	399				
Days until first living amoebae observed	MYB: 18 cond. MYB: 8				
6th vial thawed	2018–01–31				
State before freezing	Attached				
Days after freezing	461				
Days until first living amoebae observed	MYB: 12 cond. MYB: 19				

H02/13Pp	H02/13Pp	H03/14Pp	R18/15Pp	H20/16Pp	H02/13Pp	H03/14Pp
2016–12–09	2016–12–16	2017–12–14	2017–12–14	2017–12–18	2017–12–18	2017–12–19
2016–11–24	2016–11–24	2017–12–08	2017–12–08	2017–12–08	2017–12–08	2017–12–08
15	22	6	6	10	10	11
Yes	Yes	No	No	No	No	No
N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	8	5	5	5	5	5
6*500 floating, 2*500 attached	6*500 floating, 2*500 attached	600	600	600	600	600
N/A	N/A	N/A	N/A	N/A	N/A	N/A
06/01/2017	06/01/2017	31/01/2018	19/06/2019	31/01/2018	31/01/2018	19/06/2019
Attached	Attached	Attached + floating	Attached + floating	Attached + floating	Attached + floating	Attached + floating
28	21	48	552	44	44	547
N/A	N/A	MYB: 19 cond. MYB: N/A	MYB: 9 cond. MYB: 9	MYB: N/A cond. MYB: 23	N/A	N/A
2017–01–06	2017–01–06	2019–06–19	2019–06–26			2019–06–26
Floating	Floating	Attached + floating	Attached + floating			Attached + floating
30	30	552	559			554
N/A	N/A	N/A	N/A			N/A
	2018–01–31					
	Floating					
	411					
	N/A					

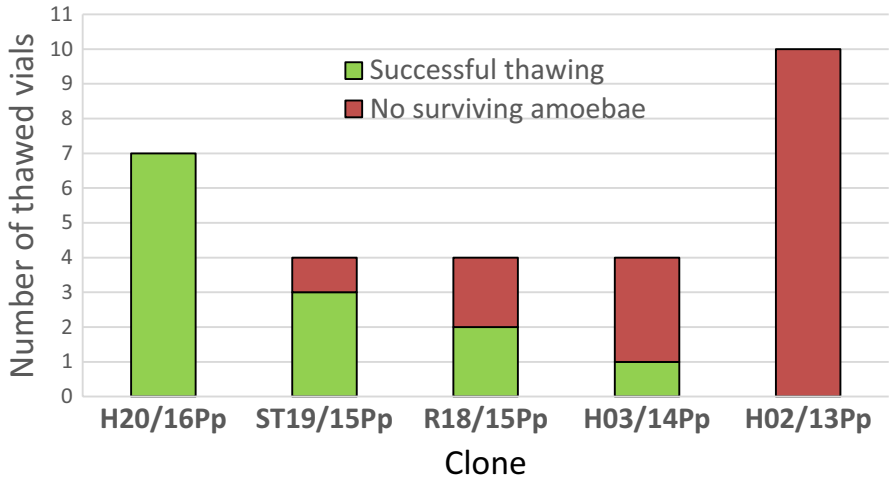

FIGURE 1 Thawing success rate, that is the percentage of successfully thawed vials relative to the total number of vials that were thawed, for five cloned isolates: H20/16Pp, ST19/15Pp, R18/15Pp, H03/14Pp and H02/13Pp

4 | CONCLUSION

In this study, we were able to thaw four out five clones of *P. perurans* after cryopreservation with success rates between 25% and 100%. The recovery rates in the successful thawing experiments were very low compared to cryopreservation of several other amoebae, and a further optimization of the method would be desirable. The results presented in this study are based on a limited number of experiments that varied in certain aspects of the methodology. However, some trends could be observed, and the presented results give a good starting point for further tests to optimize the method for cryopreservation of *P. perurans*. One of the cloned isolates, H02/13Pp, seems to be less suited for cryopreservation than the other four clones tested in this study. This was the oldest clone tested which could explain the limited success, but it is also the only of the five tested clones that was not carrying the bacterium *Candidatus* Syngnamydia salmonis, Chlamydiales (Nylund et al., 2015, 2018). The clone H03/14Pp, which we were able to recover after cryopreservation, is only three months younger than H02/13Pp, suggesting that it cannot be excluded that other factors than age of the clones could be of importance for the success of cryopreservation.

ACKNOWLEDGEMENTS

Astrid Holzer (Institute of parasitology, Biology centre CAS, České Budějovice, Czech Republic) is appreciated for sharing freezing protocols used at her laboratory for freezing *Paramoeba pemaquidensis*.

CONFLICT OF INTEREST

There is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Tröße C, Kindt M, Blindheim S, Andersen L, Nylund A. Method for cryopreservation of *Paramoeba perurans*. *J Fish Dis*. 2020;00:1–7. https://doi.org/10.1111/jfd.13295