Quantification of *Perkinsus* sp. infection intensity using Ray's Fluid Thioglycolate Medium (RFTM) Method

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Editions

Edition	Date	Updated part
1	18/02/2011	Creation

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1. Scope

This procedure explains the technique used to quantify the protistan *Perkinsus* sp. in molluscs by using a special culture medium. The present technique is adapted from Ray (1952).

2. References

- **Council Directive** 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals.
- **OIE**. Manual of Diagnostic Tests for Aquatic Animals (last edition).
- **Howard, D.W., E.J. Lewis, B.J. Keller, and C.S. Smith** (2004). Histological techniques for marine bivalves mollusks and crustaceans. NOAA Tech. Memo. NOS NCCOS 5, 218 p.
- **Villalba A., K.S. Reece, M.C. Ordás, S.M. Casas and A. Figueras** (2004). Perkinsosis in molluscs: A review. Aquat. Living Resour. 17, 411–432 (2004)
- **Ray, S.M. (1952)**. A culture technique for the diagnosis of infection with *Dermocystidium marinum* Mackin, Owell and Collier in oysters. Science, 116, 360-361.
- **Bower, S.M. (2010):** Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish: *Perkinsus* of Clams and Cockles and *Perkinsus marinus* ("Dermo" disease) of oysters. http://www.pac.dfo-mpo.gc.ca/science/species-especes/shellfish-coquillages/diseases-maladies/pages/perkincc-eng.htm

3. General information

Perkinsosis have been reported from many parts of the world (Europe, America, Asia, Australia) in different mollusc species including abalone, oysters, clams, scallops, etc. (Garcia et al. *in* Villalba, 2008).
Perkinsus olseni is a pathogenic protistan infecting different species of clams in Europe (mainly *Ruditapes decussatus* and *R. philippinarum*). It was also reported as an important pathogenic organism of the abalone *Haliotis rubra* in Australia.
Perkinsus marinus causes disease of economic importance in *Crassostrea virginica*. *Crassostrea gigas* can be infected to a lesser extent. *Perkinsus chesapeaki* is a species commonly observed in USA in several clam species (e.g. *Mya arenaria*) and the oyster *C. virginica*.

4. Equipment and medium preparation

4.1. Equipment

- Microscope with objective X10
- Haemocytometer

4.2. Medium preparation

4.2.1. Thioglycolate medium

- thioglycolate medium (Sigma T9032) : 29.4 g
- sterile sea water 800 ml

Adjust at pH 7, add enough sterile seawater for 1000 ml, and bring to the boil under stirring. Autoclave.

4.2.2. Antibiotic solution

- penicillin G (Sigma P 3032): 6.66 g
- streptomycin (Sigma S 6501): 3.20 g
- sterile sea water 1000 ml

Filter at 0.45µm and aliquote in 50 ml tubes. Keep frozen (- 20°C).

4.2.3. NaOH 2 M solution

- NaOH : 80 g
- sterile sea water 1000 ml

Keep at room temperature.

4.2.4. PBS 10 X

- NaCl : 90 g
- Anhydrous Na H_2PO_4 : 14.8 g
- KH_2PO_4 : 4.3 g
- dH_2O : 1 litre

Adjust pH to 7.2 and keep at 4°C.

5. Operating procedure

5.1. Procedure

1. Dispense 9 ml of thioglycolate medium per tube
2. Add 1 ml of antibiotic solution per tube
3. Open animals and discard internal liquid. Rinse knife and pliers with alcohol between each individual.
4. Collect the 4 gill leafs with a pinch, weight them, put them in a tube previously prepared and incubate them in the dark at room temperature for at least 1 week.
5. Centrifuge tubes at 1000 rpm for 10 minutes at room temperature
6. Discard 8 ml of supernatant
7. Add 8 ml of NaOH 2M solution and incubate at 50°C for 1 hour
8. Vortex, centrifuge tubes at 1000 rpm for 10 minutes at room temperature
9. Repeat the 2 precedent steps if tissue is not fully digested
10. Discard 8 ml of supernatant and add 8 ml of PBS 1X solution.
11. Vortex, centrifuge tubes at 1000 rpm for 10 minutes at room temperature
12. Discard most of the supernatant and keep only 2 ml of liquid
13. Mix vigorously samples before putting them on a haemocytometer
14. Count the cells under microscope (objective X10) four times on the entire haemocytometer **or** count the cells four times on 10 rectangles (see picture page 4). If number of parasites is too high, add 1 or 2 ml of PBS 1X in the tube, mix and count again.

5.2. Count

<u>After reading 10 rectangles</u>:

Number of parasites / ml = 10 000 x ΣX / 4

X: number of parasites counted

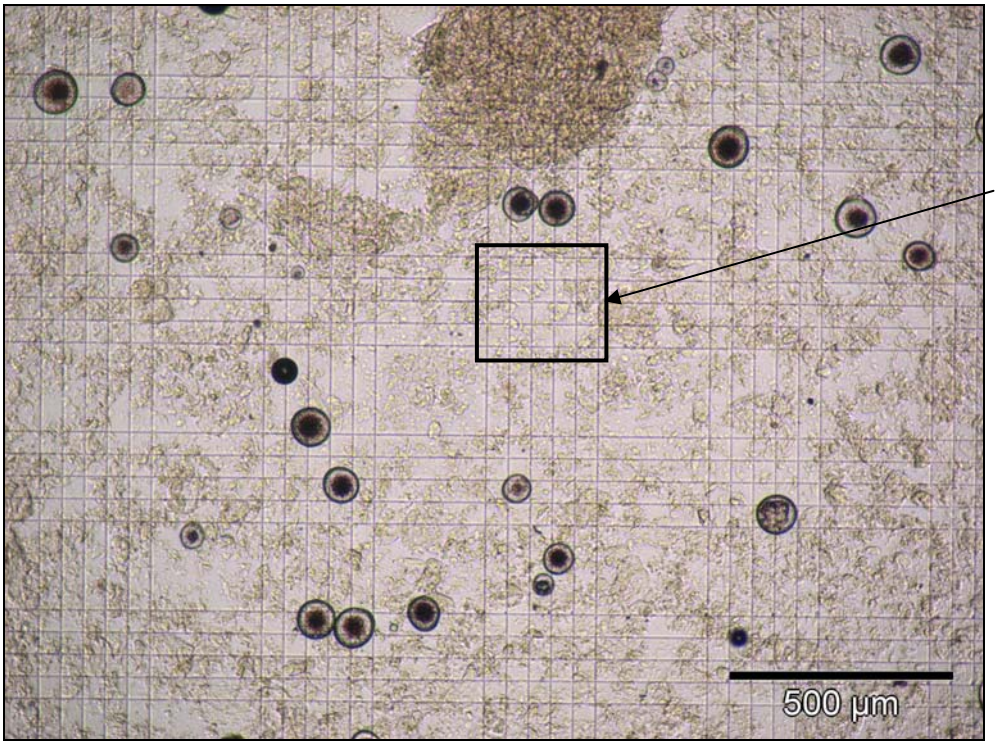
<u>After reading the entire cell</u>:

Number of parasites / ml = 1000 x ΣX / 4

X: number of parasites counted

Number of parasites per gram of tissue = Number of parasites x V / Y

V: final volume of PBS in the tube (in ml)
Y: gill weight in grams



Picture: Haemocytometer with a *Perkinsus* sp. culture in RFTM.