Marteilia refringens detection and typing by Real time Polymerase Chain Reaction

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1. Scope

This procedure explains a standard diagnostic test used for the detection and characterization of the parasite *Marteilia refringens* in bivalves including flat oysters *Ostrea edulis* and mussels, *Mytilus edulis* and *M. galloprovincialis*.
This multiplex Real-Time PCR assay amplifies a fragment of the ITS-1 (Internal Transcribed Spacer) of *Marteilia refringens* genome. It allows a specific diagnosis between *M. refringens* type O and type M as defined by Le Roux et al. (2001) by using type-specific probes.

2. References

Le Roux F., Lorenzo G., Peyret P., Audemard C., Figueras A., Vivarès C., Gouy M. & Berthe F., 2001. Molecular evidence for the existence of two species of Marteilia in Europe. J. Eukaryot. Microbiol. 48, 4: 449-454.
OIE (2014). Manual of Diagnostic Tests for Aquatic Animals, section 2.4.4, Paris, France, (http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.4.04_M_REF.pdf)

3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for real time PCR assays:

- A closed hood equipped with an UV producing system to eliminate potential contaminations when preparing real time PCR mix.
- Two complete sets of pipettes (2 µL; 20 µL; 200 µL and 1000 µL), the first one for DNA extraction and the second one for PCR mix preparation.
- An additional pipette (20 µL) to dispense samples in PCR mix.
- ARN-DNA free filter pipette tips (2 µL; 20 µL; 200 µL et 1000 µL) for DNA extraction, real time PCR mix preparation and samples dispensing.
- Real time PCR plates and associated caps.
- A thermocycler for real-time PCR (for example Mx 3000 Thermocycler sequence detector from Stratagene®/Agilent®).

Manipulator must wear a lab coat and gloves during all the different steps described below. Lab coat and gloves must be changed preferably after each main step: DNA extraction, preparation of real time PCR mix, sample dispensing and amplification. It is recommended to perform these different steps in different rooms. More particularly, amplification should take place in a separate room from DNA extraction, real time PCR preparation mix and DNA dispensing.

4. Procedure

4.1. Sample Preparation

Live or freshly dead (not decaying) oysters, which can be previously frozen, are processed for DNA extraction. DNA is extracted from digestive gland of marine bivalves.

DNA extraction can be performed by using commercial kit like the QIAamp® DNA Mini Kit (QIAGEN) and following the instructions for Tissue Test Protocol. The quality and efficacy of the extraction is controlled for example by measuring DO (260 nm) under spectrophotometer or after electrophoresis in agarose gel. DNA solutions are kept at 4°C until PCR analyses are performed.

Just before performing the real time PCR assay, samples are adjusted at a final DNA concentration of 5ng/µl.

4.2. Real Time Polymerase Chain Reaction

4.2.1. Reactives

- Brilliant III Ultrafast qPCR Master Mix (Stratagene® Catalog ref: # 600880).
- dH_20 (free of DNA and RNA)

Other commercial Q PCR Master Mix may be used if they have been demonstrated to give similar results.

4.2.2. Primers and probes

- TaqMar F 5’ GTGTTCGGCACGGGTAGT 3’
- TaqMar R 5' TGATCTGATATTATTCAGCTGTTCG 3'
- TaqProb M Texas red® 5’ GCGCTTGCCCTACGGCCGTGC 3’BHQ-2™
- TaqProb O 6-FAM 5’ GCCCTTTCCCCGACGGCCG 3’Tamra

4.2.3. Real time PCR mix

Real time PCR mix for each well is:

Product	Volume per well	Final Concentration
2X Mastermix Ultrafast Brilliant III	12,5 µl	1X
TaqMar F (20 µM)	0,13 µl	0,3µM
TaqMar R (20 µM)	0,38 µl	0,1µM
TaqProb O (20 µM)	0,31 µl	0,25µM
TaqProb M (20 µM)	0,31 µl	0,25µM
dH_2O	6,38 µl	

- Twenty (20) µl of this Real-Time PCR mix are distributed in each well of the real time PCR plate.
- Five (5) µl of DNA diluted to a concentration of 5 ng / µl are added in each well.
- Plates are sealed and centrifuged (100 *g*).

Each sample should be tested in duplicate.

4.2.4. Negatives controls

Negatives controls or NCT consist of H_20 (5 µl for 20 µl of Real-Time PCR mix). They aim at detecting potential contamination. At least one negative control should be included on each real time PCR plate.

4.2.5. Positives controls

Positive controls consist of plasmidic DNA containing the PCR target or DNA extracted from oysters or mussels known to be infected with *Marteilia refringens* type O and type M. They aim at checking that PCR reaction works. Two positive controls (one for type O and one for type M) should be included for each PCR analysis

4.2.6. Amplification

Amplification cycles are performed using a thermocycler for Real Time PCR (for example Mx 3000 Thermocycler sequence detector from Stratagene®/Agilent®) following this protocol:

- Initial denaturation: 10 min at 95°C
- Amplification: 40 cycles (30 s. at 95°C, 60 s. at 60°C).

Fluorescence is measured at the end of each cycle with ROX or Texas Red filter for *Marteilia refringens* type M and FAM filter for *Marteilia refringens* type O.

4.2.7. Results

Threshold Cycles (Ct) is defined as the cycles at which a statistically significant increase in fluorescence output above background is detected. Cts are calculated automatically by the Stratagene® thermocycler software. Reported Ct values are calculated as mean Cts of the duplicates within each reaction.

Controls

Before concluding about the status of the tested samples regarding the presence of *Marteilia refringens*, negatives controls (NTC) should not present any amplification and positive controls (types O and M) should present amplification.

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Samples

A tested sample is considered positive for *Marteilia refringens* type O if its Ct value is below 37 (≤37)
A tested sample is considered positive for *Marteilia refringens* type M if its Ct value is below 37 (≤37)
A tested sample is considered negative if there is no amplification or if its Ct value is above 37 (>37)

Remark: difference between duplicated values should not exceed 1 Ct

End