Marteilia sp. detection and characterisation by *in situ* hybridization (ISH)

Content

Editions

Edition	Date	Updated part
N° 4	April 2021	Minor protocol changes, one probe added
N°5	Decembre 2024	Creation of a general SOP for *in situ* hybridization connected with diseases-specific SOPs. This SOP was reviewed to standardise the content of diseases-specific SOPs and to include information regarding the specificity of ISH probes

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1. Scope

This procedure describes *In Situ* Hybridization (ISH) assays used for *Marteilia* sp. and *Marteilia refringens* detection to confirm a previous histological diagnosis. Two probes can be used, one probe allows the detection at the **genus level (*Marteilia* sp.)**, and another one allows the **specific detection of *Marteilia refringens and M. pararefringens***.

General steps to perform ISH assays are described in the SOP "Detection of mollusc pathogens by *In Situ* Hybridization (ISH)

This SOP provide additional information on the probes to be used for the specific detection of *Marteilia* parasites and result interpretation.

2. References

Le Roux F., Audemard C., Barnaud A. & Berthe F.C.J. (1999). DNA probes as potential tools for the detection of *Marteilia refringens*. Mar. Biotechnol., 1(6): 588–597.

Le Roux F., Lorenzo G., Peyret P., Audemard C., Figueras A., Vivarès C., Gouy M. & Berthe F.C.J. (2001). Molecular evidence for the existence of two species of *Marteilia* in Europe. J. Euk. Microbiol., 48 (4): 449–454.

WOAH (2024). Manual of Diagnostic Tests for Aquatic Animals, section 2.4. Diseases of molluscs, Chapter 2.4.0 General information and disease-specific chapters*)*: https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-manual-online-access/

3. Equipment, reagent and environmental conditions

Refer to SOP "Detection of mollusc pathogens by *In Situ* Hybridization (ISH)" and to SOP "*Marteilia refringens* detection by Polymerase Chain Reaction and species characterisation by Restriction Fragment Length Polymorphism (PCR-RFLP)" for PCR conditions used to produce the *Marteilia* probes.

4. Procedure

4.1. ISH probes

ISH probes are labelled with digoxigenin (DIG)

Targeted pathogens and gene region	Reference	Probe type	Probe name and sequence
Marteilia sp. (18S)	Le Roux et al. (1999)	Labelled PCR amplicon	Smart 2 probe: PCR product obtained with SS2/SAS1 primers (266 bp)
M. refringens type O and type M (or M. pararefringens) (ITS)	Le Roux et al. (2001)	Labelled PCR amplicon	PCR product obtained with Pr4/Pr5 (M2A/M3AS) primers (412 bp)

The smart 2 probe was tested on several bivalves infected with different *Marteilia* species, and was shown to hybridize with *M. refringens, M. pararefringens, M. cochillia, M. octospora, M. tapetis* and *Marteilioïdes*. The M2A/M3As probe hybridize with *M. refringens* and *M. pararefringens.* (EURL technical report 2021-2022).

Probe preparation (by PCR)

The probe is prepared by PCR with DNA from bivalve highly infected with *Marteilia refringens*. DNA is extracted from a piece of digestive gland.

For each probe (genus or species specific), a conventional PCR is performed as described below. The only difference with a conventional protocol is the addition of Dig labelled dUTP (Roche) in the PCR Mix.

Primers for probe preparation:

Marteilia genus (smart 2 probe),18S, Le Roux et al. 1999

Primer F SS2 5' CCG-GTG-CCA-GGT-ATA-TCT-CG 3'
Primer R SAS1 5'TTC-GGG-TGG-TCT-TGA-AAG-GC 3

Marteilia refringens, ITS Le Roux et al 2001

Primer F M2A 5' CCG CAC ACG TTC TTC ACT CC 3'
Primer R M3AS 5' CTC GCG AGT TTC GAC AAA CG 3'

PCR are prepared for a total volume of 100µl of probe. **PCR Mix** for each tube is:

	Volume per tube	**Final concentration**
Buffer (10X)*	10 µl	1X
$MgCl_2$ (25 mM)*	10 µl	2.5 mM
dNTP (2mM)	10 µl	0,2 mM
Dig Labelled dUTP (1 mM)	2.5 µl	0.025 mM
Primer F (100 µM)	1 µl	1 µM
Primer R (100 µM)	1 µl	1 µM
Taq polymérase (5U/µl)*	0,6 µl	3 U
dH_2O	65.1 µl	

- 98 µl of this PCR mix is dispensed in each PCR tube
- 2 µl of extracted DNA (100 ng/µl) is added to each tube

*Those parameters may vary depending on the commercial PCR mix used

PCR amplification

Amplification cycles are performed in a thermal cycle apparatus:

- Initial denaturation: 3min at 94°C*
- Amplification: 40 cycles (1 min at 94°C, 1 min at 55°C and 1 min at 72°C)
- Final elongation: 10 min at 72°C*

**Those parameters may vary depending on the commercial PCR mix used*

Control of the labelling efficiency

DNA sample should be amplified in parallel without the addition of DIG-labelled dUTP. PCR products with and without DIG-labelled dUTP are compared on a gel electrophoresis to control for the DIG labelling. The labelled PCR product should migrate slower and appear larger than the unlabelled PCR product.

4.2. Hybridization

Refer to SOP "Detection of mollusc pathogens by *In Situ* Hybridization (ISH)

Two types of control are used:

- **Negative** controls consist of sample tissue without probe (for each sample to analyse, prepare one slide with probe and one slide without probe).
- **Positive** controls consist of tissue sections from controlled **infected** oysters or mussels
- **The *Marteilia* sp. probe** can be used as positive control to check the integrity of DNA on analysed slides.

4.3. Interpretation

Validation of the ISH assay:

- Negative controls must appear negative.
- Positive controls must appear positive.
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Interpretation of sample results

- Slides are checked for positive signal looking like dark purple or brown spots (Figures 1 and 2).
- A positive signal with smart 2 probe in an individual detected positive by histology allows concluding that it is an infection with *Marteilia sp.*
- A positive signal with M2A/M3AS probe in an individual detected positive by histology is indicative of an infection with *Marteilia refringens* or with *Marteilia pararefringens.*

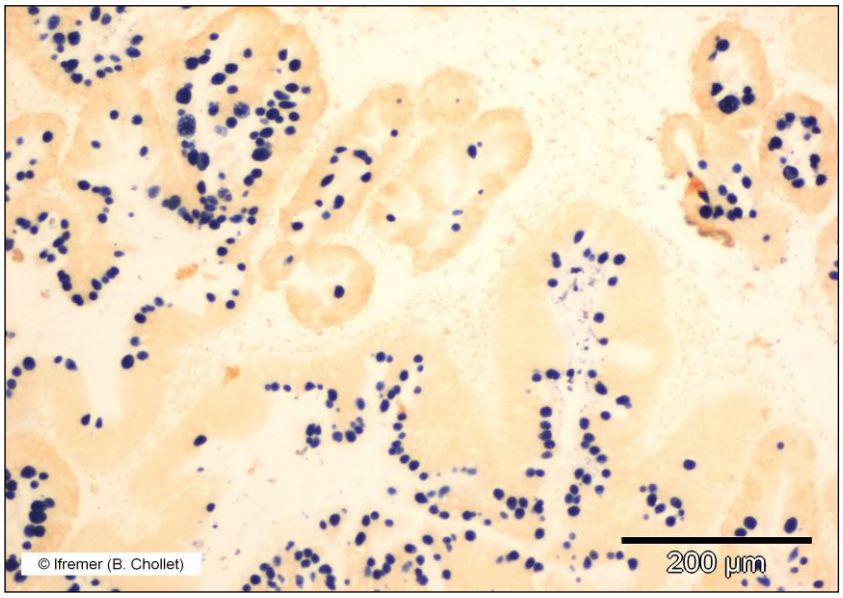

Figure 1. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the smart 2 probe: the parasites cells appear as dark spots in the epithelia of the digestive tubules.

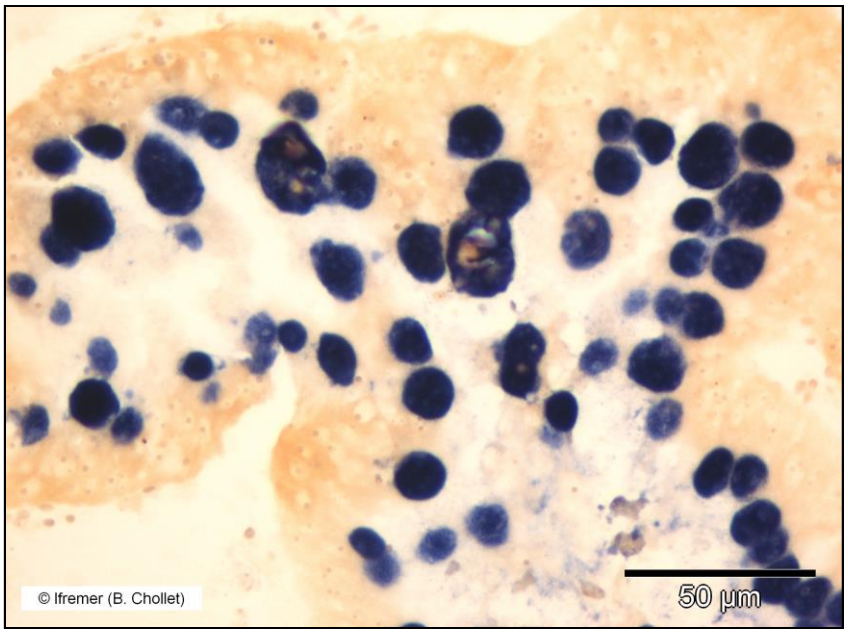

Figure 2. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the smart 2 probe: the parasites cells appear as dark spots in the epithelia of the digestive tubules.

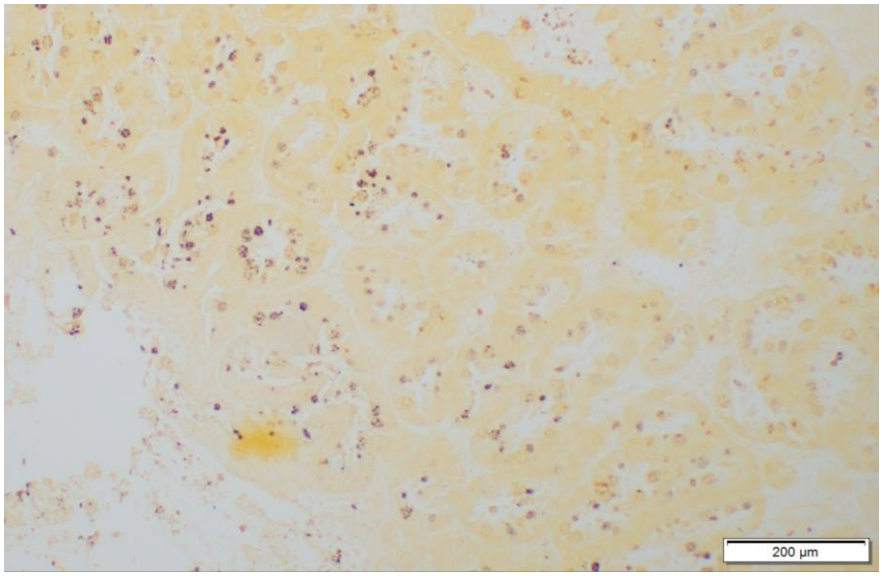

Figure 3. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the M2A/M3AS probe: the parasites nuclei appear as dark brown spots in the epithelia of the digestive tubules (Picture B. Chollet)

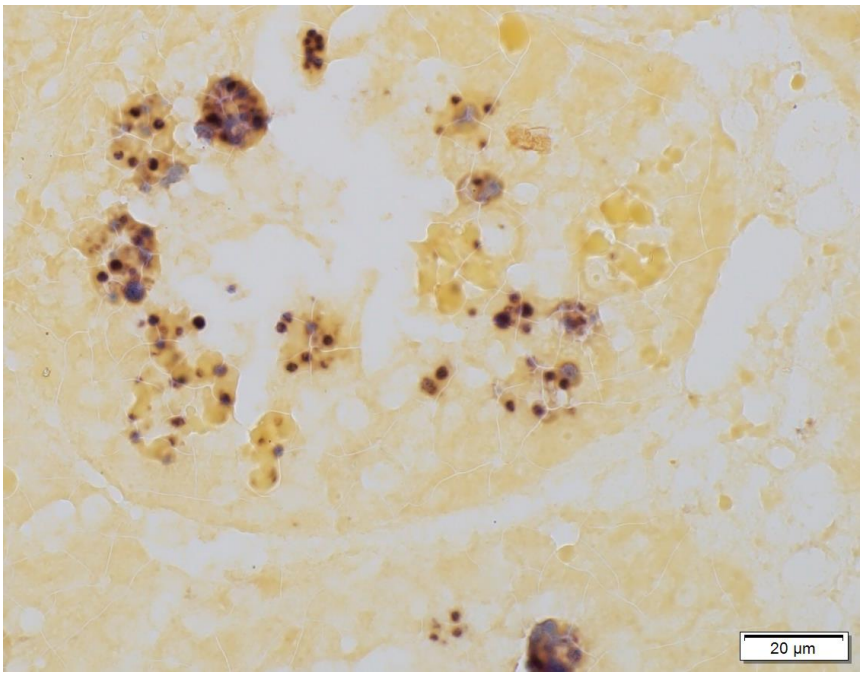

Figure 4. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the M2A/M3AS probe: the parasites nuclei appear as dark brown spots in the epithelia of the digestive tubules (Picture B. Chollet)