Marteilia refringens detection by *in situ* hybridization (ISH)

Adapted from Le Roux *et al*. (1999, 2001)

Content

Editions

Edition	Date	Updated part
N° 4	April 2021	Minor protocol changes, one probe added

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1. Scope

This procedure explains a standard diagnostic test used for *Marteilia refringens* detection to confirm a previous histological diagnosis at the genus level. 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***. This procedure do not includes the preparation of histological slide.

Important note: The different steps of the preparation of the histological slides are explained in the procedure "Molluscs processing for diagnosis by histopathology". The only differences are the use of some aminoalkylsilane coated slides instead of classical histological slides and the thickness of the tissue section (5 µm instead of 2 µm). The different steps of the preparation of the probe by PCR are explained in chapter 4.1. The only difference with a conventional PCR is the addition of dUTP in the PCR Mix.

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.

OIE (2019). Manual of Diagnostic Tests for Aquatic Animals, section 2.4.4, Paris, France. *See the Manual of Diagnostic Tests (online)*: http://www.oie.int/en/international-standard-setting/aquatic-manual/access-online/

Commission implementing decision *2015/1554* laying down rules for the application of Directive 2006/88/EC as regards requirements for surveillance and diagnostic methods

3. Equipment and environmental conditions

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

- A closed hood equipped with an UV producing system to eliminate potential contaminations when preparing PCR mix
- Two complete sets of pipettes (2 µl; 20 µl; 200 µl and 1000 µl) for PCR mix preparation and for the different *in situ* hybridization steps.
- Three different pipettes: one pipette (2 µl) to dispense DNA from a bivalve infected with *Marteilia refringens* in PCR mix, one pipette (20µl) for BET sampling and another pipette (20 µl) to load PCR products in agarose gels
- Filter pipette tips (2 µl; 20 µl; 200 µl and 1000 µl) for PCR mix preparation, DNA dispensing and hybridization mix dispensing.
- Pipette tips (20 µl; 200 µl and 1000 µl) to collect BET and to load amplification products in agarose gel, to collect and dispense Proteinase K suspension, probes and buffers on slides
- A thermal cycler to perform amplifications
- A horizontal electrophoresis system for PCR products electrophoresis
- An UV table to observe PCR products after agarose gel electrophoresis
- A system to acquire pictures of the gels
- A humid chamber to maintain slides in humid conditions
- A heated slide moat to denature target DNA present in slides
- An oven to maintain slides during hybridization step at 42°C and under gentle shaking
- Paper towelling
- Racks for histological slides

- Aminoalkylsilane coated slides (SIGMA)
- Hybridization chamber (ABgene) to be placed on slides during probe and antibody incubation steps
- A timer

Manipulator must wear a lab coat and some gloves during all the different steps described bellow. Lab coat and gloves must be changed preferably after each main step of the PCR: preparation of PCR mix, DNA dispensing, amplification and gel loading. It is recommended to perform these different steps in different rooms. Amplification and gel loading/electrophoresis in particular should take place in a room separate from PCR mix preparation and DNA dispensing. **Hybridization and revelations steps must be performed under a fume hood**.

4. Reagents and suppliers

Product	Supplier	Reference
Anti-Digoxigenin-AP, Fab fragments	Roche	11 093 274 910
Bismark brown Y	Sigma	B2759
Blocking reagent	Roche	11096176001
Denhart 50X	Euromedex	EU0505A
Dextran sulfate	Euromedex	1020A
Dig dUTP	Roche	11 093 088 910
EDTA	Sigma	E5134
Dionized formamide 100%	Euromedex	1117
Maleic acid	Sigma	M0375
$MgCl_2$	Sigma	M0250
NaCl	Euromedex	1112A
NBT/BCIP	Roche	11681451001
Proteinase K	Euromedex	EU 0090-B
SSC 20X	Euromedex	EU 0300-A
t ARN	Sigma	R8508
Tris	Sigma	T1503

And classical reagent to perform classical PCR (to produce the HIS probe):

- 5 X Buffer (furnished with the Taq DNA polymerase)
- $MgCl_2$ (furnished with the DNA polymerase) (25 mM)
- Taq DNA Polymerase (for example Go Taq,Promega # M3001) 5 U/µl
- dNTP Master Mix (20mM)
- H_20 (free of DNA and RNA)
- primers and probes

5. Procedure

5.1. 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 using QIAamp® DNA Mini Kit (QIAGEN) and following instructions for Tissue Protocol.

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'

Note: analytical specificity of *M. refringens* probes has never been evaluated

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 X°C* and 1 min at 72°C)
- Final elongation: 10 min at 72°C

*Hybridization of the primers: X = 60°C for SS2/SAS1, X= 55°C for M2A/M3AS

5.2. Hybridization

5.2.1. Reactives

- Xylene
- Ethanol 100 %
- Ethanol 95 %
- TE :

Tris base 1 M	50 ml
EDTA 0.5M, pH 8	20 ml
NaCl 5 M	2 ml
Distilled water for 1 l	

- Proteinase K initial solution at 10 mg/ml in TE is used diluted 100 X

- SSC 20X :

NaCl (0.3 M)	88.2 g
NaCl (3 M)	175.3 g
Distilled water for 1 l	
Ajust pH at 7	

- Hybridization buffer:

dextran sulfate	1 g
SSC 20X	2 ml
tARN from yeast at 10 mg/ml	150 µl
Denhart 50 X	200 µl
Distilled water for a final volume of 5 ml	
Heat the suspension and finally add:	
Deionized formamid at 100 %	5 ml

- Buffer Dig 1:

Maleic acid	11.61 g
NaCl 5M	30 ml
Distilled water for 1 l	
Adjust at pH 7.5 (by adding about 15.5 g of NaOH)	
Autoclave	

- Buffer Dig 2:

Blocking reagent	1 g
Buffer Dig 1 for 100 ml	
Mix at 50°C	
Kept at –20°C	

- Buffer Dig 3:

Tris 1 M, pH 8	50 ml
NaCl 5 M	10 ml
$MgCl_2$ 1 M	25 ml
Distilled water for 500 ml	
Ajust at pH 9.5	
Autoclave	

- Buffer Dig 4:

Tris 1 M, pH 8	5 ml
EDTA 0.5 M, pH 8	1 ml
Distilled water for 500 ml	
Autoclave	

- Detection solution:

NBT/BCIP (67 % in DMSO, v/v)	20 µl
Buffer Dig 3	1 ml

5.2.2. Controls

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

5.2.3. Deparaffinization

1. Sections are immersed twice in xylen for 10 min.
2. Sections are dehydrated by two successive immersions in ethanol 100% for 10 min.
3. Sections are air dried.

5.2.4. Deproteinization

1. 200 µl of proteinase K (100 µg/ml in TE) are displayed on each section.

2. Slides are incubated for 15 min at 37°C in a humid chamber.
3. Slides are dehydrated in ethanol 95 % for 1 min and then in ethanol 100 % for 1 min.

5.2.5. Hybridization

1. Adhesive frames (ABgene) are fixed on each slide
2. 100 µl of a mix consisting of hybridization buffer and digoxigenin labelled probe (between 5 and 10 ng /100µl of buffer) are spread inside the frame on the tissue.
3. Sections are covered with plastic coverlids.
4. Slides are denaturated for 5 min at 94°C and cooled on ice.
5. Slides are then incubated at 42°C in a humid chamber overnight

5.2.6. Washing and detection

- Adhesive frames are deleted.
- Slides are immersed in SSC 2X at room temperature twice for 5 min.
- Slides are immersed in SSC 0.4X at 42°C for 10 min.
- Slides are immersed in buffer Dig 1 for 1 min.
- 200 µl of buffer Dig 2 are spread on the tissue and slides are incubated in a humid chamber for 30 min at room temperature.
- 200 µl of a mix consisting of buffer Dig **2** and anti-digoxigenin- alkaline phosphatase conjugate (1:500 in buffer Dig **2**) are added on the sections
- Slides are incubated in a humid chamber at room temperature for 1 h.
- Slides are washed in buffer Dig 1 twice for 1 min.
- Slides are immersed in buffer Dig 3 for 10 min
- 200 µl of detection solution are added on each slide
- Slides are incubated for 30 minutes in the dark or more if the positive control does not show positive signal
- Reaction is stopped by immersion in buffer Dig 4

5.3. Counter staining and mounting

5.3.1. Reactives

- Bismark brown Y

Bismark brown Y	0.5 g
Ethanol at 30 %	100 ml
Filter the solution on a coffee filter	

- Ethanol 100
- Ethanol 95
- Xylene
- Mounting medium (e.g. Eukitt® resin)

5.3.2. Counter staining

1. Slides are immersed in Bismark brown Y for 1 min
2. Slides are rinsed under tap water
3. immersed in ethanol 95 and 100 30 sec for each

5.3.3. Mounting

1. Slides are rinsed 10 to 30 sec in Xylene
2. A drop of mounting medium (e.g. Eukitt® resin) is added to mount the cover-slip
3. Slides are observed under a light microscope

6. Interpretation

- Negative controls must appear negative.
- Positive controls must appear positive.

- 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 allows concluding that it is an infection with *Marteilia refringens.*

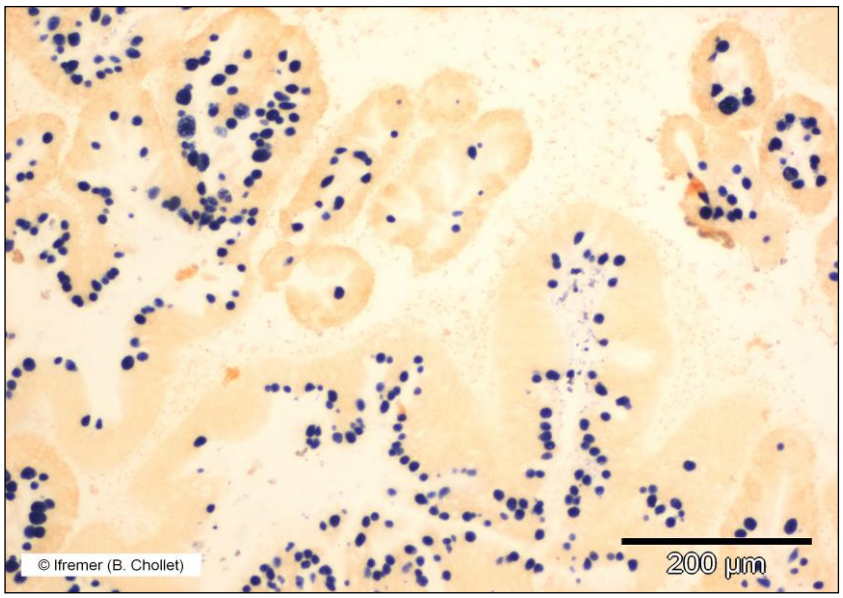

Figure 1. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the smart 2 probe: the parasites cells appear as dark spots in the epithelia of the digestive tubules.

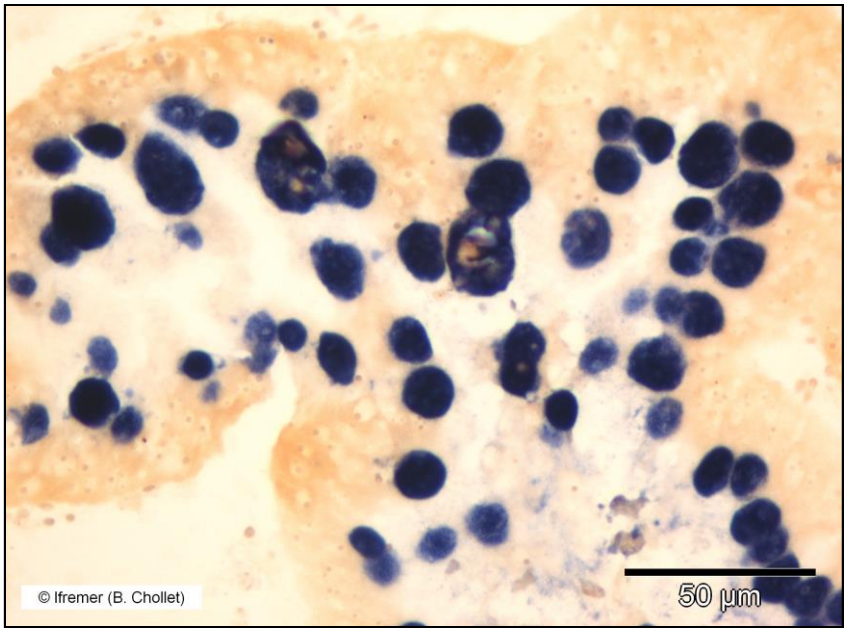

Figure 2. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the smart 2 probe: the parasites cells appear as dark spots in the epithelia of the digestive tubules.

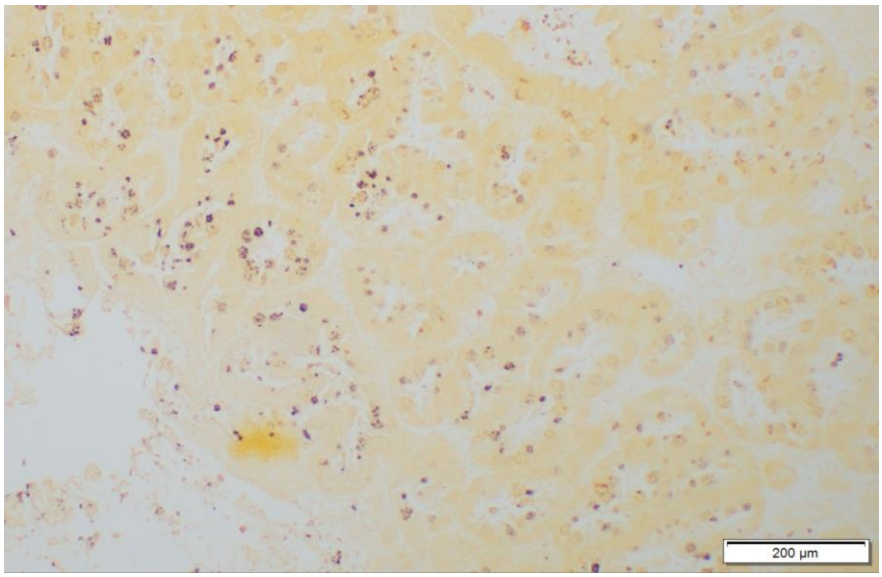

Figure 3. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the M2A/M3AS probe: the parasites nuclei appear as dark brown spots in the epithelia of the digestive tubules (Picture B. Chollet)

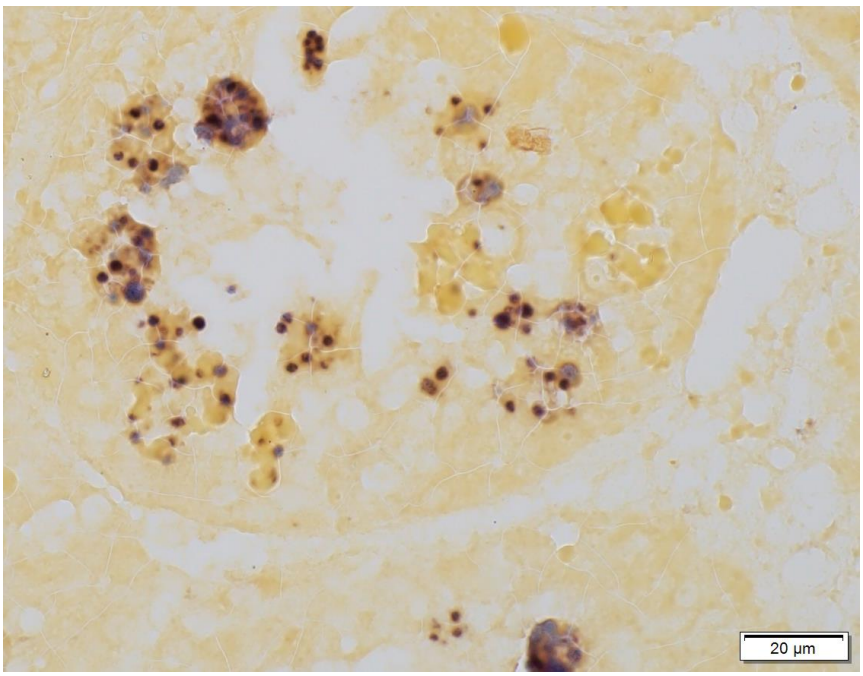

Figure 4. Flat oyster *Ostrea edulis* infected with *Marteilia refringens* tested by *in situ* hybridization with the M2A/M3AS probe: the parasites nuclei appear as dark brown spots in the epithelia of the digestive tubules (Picture B. Chollet)