ARTIFICIAL DIGESTION OF FISH FILLET

Standard Operating Procedure (SOP)

SUMMARY

1. SCOPE

This Standard Operating Procedure provides instructions to perform artificial digestion of marine and freshwater fish fillets for the isolation of Anisakidae and Opisthorchiidae larval stages present in muscular tissue.

2. INTRODUCTION

Marine and freshwater fishes are intermediate hosts for various parasites that can cause severe diseases in humans, if ingested by eating raw or undercooked fish. L3 larval stage of nematodes of the family Anisakidae, belonging to the genus *Anisakis* and *Pseudoterranova*, infect a large panel of marine fish and cephalopods and can cause anisakiasis in humans. The metacercaria stage of trematodes of the family Opisthorchiidae (including the species *Clonorchis sinensis, Opisthorchis viverrini* and *Opisthorchis felineus*), Heterophyidae and Echinostomatidae, infect freshwater fish and can cause opisthorchiasis/clonorchiasis, and other trematode infections.
The artificial digestion of the fish flesh is the method employed to detect nematode larvae, or trematode metacercariae in fish fillets. This method allows to collect the parasites, and then to carry out: i) morphological and/or molecular identification at the species/genotype level; ii) *in vitro* culture; iii) infection of laboratory animals.

3. REFERENCES

Reg. (CE) n. 852/2004 of 29/4/2004 and its later amendments
Reg. (CE) n.853/2004 of 29/4/2004 and its later amendments
CODEX STAN 244-2004 Standard for salted Atlantic herring and salted sprat. Joint FAO/WHO Food Standards Programme.

4. EQUIPMENT

4.1. Thermometer 1-100 °C;

4.2. analytical balance;

4.3. stereo-microscope with a substage transmitted adjustable light source (magnification 15-20X);

4.4. blender with a sharp chopping blade;

4.5. magnetic stirrer with an adjustable heating plate;

4.6. stainless steel sieve, mesh size approx. 500 microns (for Opistorchidae) or 1000 microns (for Anisakidae);

4.7. tubes or measuring cylinders (50 or 100 ml plastic or glass);

4.8. glass beakers;

4.9. conical glass separatory funnels;

4.10. pipettes (1, 10 and 25 ml);

4.11. scissors or sharp knives;

4.12. Teflon-coated stir bar;

4.13. Heating cabinet (30-50°C), optional (see paragraph 6.5).

5. REAGENTS

5.1. Tap water heated to 40-45°C;

5.2. hydrochloric acid;

5.3. pepsin powder 1: 10,000 NF (US National Formulary) corresponding to 1: 12,500 BP (British Pharmacopea) and to 2,000 FIP (Fédération Internationale de Pharmacie) or liquid pepsin 660 EP (European Pharmacopoeia) unit/ml;

5.4. 90% ethanol.

6. PROCEDURE FOR ANISAKIDAE

If you start from fish fillet, go to point 6.2.

6.1. Skin and eviscerate the fish, collect the muscular tissue;

6.2. Add to a glass beaker in the following sequence: an appropriate volume of tap water preheated to 40-42°C, 25% hydrochloric acid and pepsin according to Table 1.

Table 1. Digestion solution preparation.

Fish fillet	**Water**	**HCl 25% or 37%**	**Pepsin powder (liquid)***
100g	2L	If 25%: 16 ml ± 0.5 If 37%: 10.8 ml ± 0.5	10 g ± 0.2 g (30 ml)

***1g pepsin powder = 3ml liquid pepsin**

6.3. Chop the fish fillet by scissors and knives (or alternatively in the blender for 1-2 seconds by adding a small volume of digestion solution);

6.4. Transfer the minced tissues into the beaker and add the digestion solution (including the remaining solution if blender has been used) and add the stirring rod;

6.5. Place the beaker on the magnetic stirrer and set the heating plate at 40-42°C, cover the glass beaker with an aluminum layer to avoid evaporation and spurts. Alternatively, place the magnetic stirrer, without heating, in a heating cabinet set a 42°C ± 2;

6.6. Incubate the solution under stirring condition until the tissue disappears (approximately 15-20 min, digestion time should be adjusted according to the flesh consistency), covering the glass beaker with aluminium foil to keep a constant temperature, decrease evaporation and avoid splashing

6.7. Switch off the stirrer and pour carefully (to avoid overflow) the digestion solution through the sieve into a beaker;

6.8. Anisakidae larvae can be detected on the sieve, collected and examined under the stereomicroscope with transmitted light for morphological identification, or processed for further analysis.

The larvae can be transferred in a vial filled with 90% ethanol and stored at a temperature range between -20°C and 10°C up to 5 years.

7. PROCEDURE FOR OPISTHORCHIIDAE

If you start from fish fillet, go to point 7.2.

7.1. Skin and eviscerate the fish, collect the muscular tissue;
7.2. Add to a glass beaker in the following sequence: an appropriate volume of tap water preheated to 40-42°C; 25% hydrochloric acid and pepsin according to Table 2

Table 2. Digestion solution preparation.

Fish fillet	**Water**	**HCl 25% or 37%**	**Pepsin powder (liquid)***
100g	2L	If 25%: 16 ml ± 0.5 If 37%: 10.8 ml ± 0.5	10 g ± 0.2 g (30 ml)

***1g pepsin powder = 3ml liquid pepsin**

7.3. Chop the fish fillet by scissors and knives and then homogenate it in the blender for 5 seconds by adding a small volume of digestion solution;
7.4. Transfer the blended tissues into the beaker together with the remaining digestion solution and add the stirring rod;
7.5. Place the beaker on the magnetic stirrer and set the heating plate at 40-42°C, cover the glass beaker with an aluminum layer to avoid evaporation and spurts. Alternatively, place the magnetic stirrer, without heating, in a heating cabinet set a 42°C ± 2;
7.6. Incubate the solution under stirring condition until the tissue disappear (approximately 15-20 min, digestion time should be adjusted according to the flesh consistency), covering the glass beaker with aluminium foil to keep a constant temperature, decrease evaporation and avoid splashing (;
7.7. Switch off the stirrer and pour carefully (to avoid overflow) the digestion solution through the sieve into the sedimentation funnel;
7.8. Leave the solution to sediment for 30 minutes, open the stopcock to recover 40 ml of the solution in a centrifuge tube or in a glass measuring cylinder;
7.9. Leave the solution to sediment in the tube/cylinder for at least 5 minutes then aspirate supernatant up to 10 ml;
7.10. Pour the 10 ml pellet in a Petri dish and analyze the sediment under the stereomicroscope at 15-20 magnification to detect Opisthorchiidae metacercariae.

The metacercariae can be transferred in a vial filled with 90% ethanol and stored at a temperature range between -20°C and 10°C up to 5 years.

8. SAFETY MEASURES

Laboratory staff performing the procedure, shall wear disposable gloves, mask and lab coat. The use of a liquid pepsin formulation may be advantageous as it could reduce the risk of an occupational allergic reaction in lab staff.