ARTIFICIAL DIGESTION OF FISH FILLETS FOR THE ISOLATION OF Anisakidae AND Opisthorchidae LARVAL STAGES

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 Opisthorchidae larval stages present in muscular tissues.

2. INTRODUCTION

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

3. REFERENCES

Reg. (CE) n. 852/2004 of 29/4/2004
Reg. (CE) n.853/2004 of 29/4/2004
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 180-1000 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.

5. REAGENTS

5.1. tap water heated to 40-45°C;

5.2. hydrochloric acid (25%, molar concentration: 7.8-7.9);

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, wash the carcass with tap water and collect the muscular tissues;

6.2. Add to a glass beaker in the following sequence: an appropriate volume of tap water preheated to 46-48°C; 25% hydrochloric acid and pepsin according to Table 1.

Table 1. Digestion solution preparation.

Fish fillet	Water	HCl 25%	Pepsin powder (liquid)*
100g	2L	16 ± 0,5 ml	10 ± 0.2 g (30 ml)

*1g pepsin powder = 3ml liquid pepsin

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

6.4. Transfer the blended or chopped tissues into the beaker together with the remaining digestion solution and add the stirring rod;

6.5. Place the beaker on the magnetic stirrer and set the heating plate at 40-42°C;

6.6. Incubate the solution under stirring condition until the tissue disappear (approximately 15-20 min), covering the glass beaker with aluminium foil to keep a constant temperature and decrease evaporation;

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 their morphological identification or processed for further analyses.

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 TREMATODE METACERCARIAE

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

7.1. Skin and eviscerate the fish, wash the carcass with tap water and collect the muscular tissue;
7.2. Add to a glass beaker in the following sequence: an appropriate volume of tap water preheated to 438-40 °C; 25% hydrochloric acid and pepsin according to Table 2

Table 2. Digestion solution preparation.

Fish fillet	Water	HCl 25%	Pepsin powder (liquid)*
100g	2L	10 ± 0,5 ml	10 ± 0.2 g (30 ml)

*1g pepsin powder = 3ml liquid pepsin

7.3. Chop fish fillet by scissors and knives and then homogenate in the blender for 1-2 seconds at the most 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 45°C;
7.6. Incubate the solution under stirring condition until the tissue disappear (approximately 20-25 min), covering the glass beaker with aluminium foil to keep a constant temperature and decrease evaporation;
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, then 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 metacercariae.

It is important to stress that freshwater fish can harbor in their muscle tissues many species of metacercariae without zoonotic importance.

Metacercariae can be identified at the genus and/or species level by their morphology but a great experience in the specific field is requested.

Alternatively, 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 for their molecular identification.

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 allergic reaction in lab staff.