Standard Operating Procedure (SOP)

Title: Culture of *Renibacterium salmoninarum* from Tissues and Ovarian Fluid

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Purpose: Culture of *Renibacterium salmoninarum* in fish tissues or ovarian fluids

Sections:

I. Important considerations before starting
II. References
III. Preparation of media and reagents
IV. Culture procedure for kidney tissue
V. Culture procedure for ovarian fluid

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I. Important considerations before starting

Glassware: It is advisable to dedicate a separate set of glassware for culture to avoid antigen contamination of glassware used for other assays. This is most important in laboratories that also use immunological methods to detect *R. salmoninarum* antigen (e.g. ELISA). Extracellular proteins of the bacterium, especially the p57 antigen, can remain on container surfaces after conventional detergent washing and autoclaving.

pH meter: Check the pH of the medium before adding nurse medium to avoid contaminating probe with *R. salmoninarum* antigen.

II. References

Evelyn, T.P.T. 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. Bulletin of the Office of International Epizootics 87: 511-513.

Evelyn, T.P.T., L. Prosperi-Porta, and J.E. Ketcheson. 1990. Two new techniques for obtaining consistent results when growing *Renibacterium salmoninarum* on KDM2 culture medium. Diseases of Aquatic Organisms 9: 209-212.

Jansson, E., T. Hongslo, J. Höglund, and O. Ljungburg. 1996. Comparative evaluation of bacterial culture and two ELISA techniques for the detection of *Renibacterium salmoninarum* antigens in salmonid kidney tissues. Diseases of Aquatic Organisms 27:197-206.

III. Preparation of media and reagents

SKDM2: Recipe for 1 liter of selective kidney disease medium-2 (SKDM) agar plates (recipe makes approximately fifty 100 x 15 mm plates).

- Add measured amounts to a 2L flask
  - Peptone 10 g
  - Yeast extract 0.5 g
  - Cysteine-HCL* 0.5 g
  - Deionized water 900 mL
- Mix with stir bar and adjust medium to pH 6.5
- Add 15 mL of thawed nurse medium
- Add agar
  - Agar 18g
- Autoclave medium on liquids cycle for 30-40 minutes at 15 psi and 121°C.
- Reduce temperature of the sterilized medium to 50°C
- Add 100 mL of fetal bovine serum (FBS)
- Add antibiotics (See Table 1: volume needed for 1 L)
- Dispense 20 mL agar per plate using a sterile disposable pipet
- Let plates cool for 15-20 minutes
- Seal plates into culture plate sleeve or plastic zipper bags and store in 15°C medium cabinet

Table 1: Antibiotics for SKDM2

Antibiotic	Stock solution[1]	Final w/v	**Vol needed for 1 L**
Cyclohexamide	1 g in 100 mL dH20[2]	0.005%	**5 mL**
D-cycloserine	125 mg in 10 mL dH20	0.00125%	**1 mL**
Polymyxin-B sulfate	125 mg in 10 mL dH20	0.0025%	**2 mL**
Oxolinic acid	25 mg in 10 mL of 0.05N NaOH[3]	0.00025	**1 mL**

[1] Smaller volumes can be made. Antibiotics should be filter-sterilized (0.2 µm filter) before use.

[2] Cyclohexamide can be stored at 4°C but other antibiotics should be made fresh each time the medium is prepared.

[3] To make 0.05N NaOH: add 2.5 mL 1N NaOH in a 50 mL sterile volumetric flask and add sterile nH2O to 50 mL mark.

*Note: The original protocol by Evelyn (1977) used 1 g/L cysteine. This concentration tends to result in crystal formation in the medium. Equivalent growth of *R. salmonarum* is attained *without* crystal formation if the cysteine is reduced to 0.5 g/L.

PBS-peptone: Recipe for 1 liter of 1X Voller phosphate buffered saline (PBS) pH 7.4 + 0.1% peptone.

- Add measured amounts into a 2 L bottle
  - NaCl 8.0 g
  - KH_2PO_4 0.2 g
  - Na_2HPO_4 anhydrous 1.09 g
  - KCl 0.2 g
  - Peptone 1.0 g
  - Deionized H_2O to 1.0 L
- Mix with stir bar and check to make certain that pH is 7.4
- Autoclave on liquids cycle for 30-40 minutes at 15 psi and 121°C, then store in refrigerator at 4°C

Nurse Medium: Recipe for 1 liter of spent KDM-2 broth for use as a metabolite.

- Inoculate 1 liter of KDM 2 broth medium (recipe provided below) with 1 mL of stock *Renibacterium salmoninarum* isolate (ATCC 33209)
- Allow the culture to grow at 15° C for 10-14 days while being stirred
- Subject culture to centrifugation at 5000 x g for 20 minutes at 4°C
- Pool the supernatant and filter through a 0.2 µm bottle filter for sterilization.
  - This may take several filters, depending on the amount of metabolite desired and how well the cells were separated out by centrifugation
- Dispense into 15 to 30-mL aliquots and freeze at -80° C

KDM2: Recipe for 1 liter of kidney disease medium-2 (KDM2) broth.

- Add measured amounts to a 2L flask
  - Peptone 10 g
  - Yeast extract: 0.5 g
  - Cysteine-HCL* 0.5 g
  - Deionized water 900 mL
- Mix with stir bar and adjust medium to pH 6.5
- Add thawed nurse medium
  - Nurse medium 15 ml
- Autoclave medium on liquids cycle for 30-40 minutes at 15 psi and 121°C., then store in refrigerator at 4°C
- Reduce temperature of the sterilized medium to 50°C
- Add fetal bovine serum (FBS)
  - FBS 100 mL
- Store in a 15°C medium cabinet or a 4°C refrigerator

*Note: The original protocol by Evelyn (1977) used 1 g/L cysteine. This concentration tends to result in crystal formation in the medium. Equivalent growth of *R. salmonarum* is attained *without* crystal formation if the cysteine is reduced to 0.5 g/L.

IV. Culture procedure for kidney tissue

Notes:

- *Keep tissue cold during entire procedure.*
- *Use freshly harvested kidney tissue.*

Method:

1. **Put the kidney sample into a pre-weighed sterile bag with a top closure**.

2. **Homogenize tissue with a heavy rolling pin or stomacher**. Homogenize small kidneys in a tube with a sterile pestle or other implement.

3. **Determine the tissue weight**.
   Example: kidney tissue = 0.1 gram

4. **Add PBS-peptone at 10mL / gram tissue.** Mix tissue and PBS-peptone in bag by kneading the bag with fingers or by compression with a roller.
   Example: 1ml for 0.1 gram tissue

5. **Transfer to a sterile centrifuge tube and subject to 2500 x *g* centrifugation for 20 minutes at 4°C**.

6. **Discard supernatant**.

7. **Resuspend pellet in PBS-peptone at 1:1 (w/v) ratio**. Vortex well.
   Example: 0.1 gram of tissue would be resuspended in 100 µl (0.1 mL) PBS peptone

8. **Spread 10 µL of homogenate over entire surface SKDM2 agar plates with a sterile bent glass rod**. Alternatively, subject homogenates to serial 10-fold dilution in PBS-peptone for quantitative culture.

9. **Incubate at 15°C for up to 12 weeks**. Closure of plates with paraffin film or placement in a plastic zipper bag will retard drying.

10. **The presence of *R. salmoninarum* is typically confirmed by fluorescent antibody test (FAT) of colonies showing characteristic morphology**. See BACT-3 SOP for DFAT.

11. **Calculate CFU / g.** Divide the number of colonies by gram of tissue plated (take into account any further dilution for quantitative culture).
    Example: If the starting tissue was 0.1 gram and 10 µl of homogenate was spread, than 0.01 g were used. So 10 colonies / 0.01 g = $1x10^3$ CFU/g

V. Procedure for Ovarian Fluid

Notes:

- *Keep ovarian fluid cold during entire procedure.*
- *Use freshly harvested ovarian fluid.*

Methods:

1. **Spread 100 µl of ovarian fluid over entire surface of SKDM2 agar plate with a sterile bent glass rod**. Alternatively, subject homogenates to serial 10-fold dilution in PBS-peptone for quantitative culture.

2. **Incubate at 15°C for up to 12 weeks**. Closure of plates with paraffin film or placement in a plastic zipper bag will retard drying.

3. **The presence of *R. salmoninarum* is typically confirmed by fluorescent antibody test (FAT) of colonies showing characteristic morphology**. See BACT-3 SOP for DFAT.

4. **Calculate CFU / mL.** Divide the number of colonies by mL of tissues plated (take into account any further dilution for quantitative culture).

   Example:
   If the starting volume was 0.1 mL and 100 colonies were observed:
   100 colonies / 0.1 mL = $1x10^3$ CFU/mL
   If the ovarian fluid was diluted 1:10 before plating, then:
   *(100 colonies / 0.1 mL) * 10 fold dilution = $1x10^4$ CFU/mL*