



UNIVERSITEIT  
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Laboratory of Microbiology  
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# **SOP**

## **Standard Operating Procedure**

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Acronym:

**ASIARESIST-MIC**

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Title:

### **ANTIBIOTIC SUSCEPTIBILITY TESTING OF AQUACULTURE-ASSOCIATED BACTERIA WITH THE BROTH MACRODILUTION METHOD (MIC DETERMINATION)**

References:

**see section C**



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**Reviewed & approved by:** all ASIARESIST partners

**ANTIBIOTIC SUSCEPTIBILITY TESTING OF AQUACULTURE-  
ASSOCIATED BACTERIA WITH THE BROTH MACRODILUTION METHOD  
(MIC DETERMINATION)**

**A. PURPOSE & PRINCIPLE**

The purpose of this SOP is to describe the procedure for determining the Minimal Inhibitory Concentration (MIC) for a number of antibiotics with particular relevance in aquaculture activities. In contrast to the disc diffusion method, which is a ‘semi-quantitative’ method, determination of MIC values provides a quantitative measure for the level of resistance expressed by the test organism. Next to the agar dilution method and the Etest, the broth dilution method is one of the most frequently used methods to determine MIC’s. The method is based on the inoculation of a standardized liquid culture of the test strain in a dilution series of the antibiotic for which the MIC is determined. The first concentration in the dilution series at which no visual growth can be determined is then considered as the MIC. Both NCCLS (in Mueller-Hinton broth) and BSAC (in Iso-sensitest broth) have proposed qualitative criteria to classify organisms as susceptible or resistant on the basis of MIC values.

**B. METHOD DESCRIPTION**

NOTE 1: This protocol was written to perform MIC determinations in glass tubes (= macrodilution). However, some partner labs may prefer to use microtiter plates and automated micro-inoculator systems to minimize antibiotic use (= microdilution). In this case, technical adaptations should be introduced in this SOP-MIC by the individual partners, hereby respecting the experimental conditions described below (e.g. volumes may change but concentrations should be remain unchanged).



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NOTE 2: This version of the protocol was written to determine the MIC of chloramphenicol. Depending on the disc diffusion susceptibility testing, the protocol may/should be extended with other antibiotics.

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### I. Bacterial cultivation and material preparations (DAY 1-2)

- The organism to be tested should be subcultured using a suitable medium under optimal incubation conditions to obtain a fresh overnight grown culture. As standard conditions, growth in Iso-Sensitest Agar (ISA; Oxoid) and Broth (ISB) at 28 °C under aerobic atmosphere are recommended. For subculturing, remove one bead from the cryovial and streak out onto an ISA plate. Incubate at 28°C overnight (or longer until clear visible growth is observed).
- After overnight incubation, the streak cultures are checked for purity. A number of pure colonies (app. 5 or more if the isolate is a suspected fastidious organism) are introduced into a glass culture tube containing 10 mL ISB and incubated at 28°C overnight (or longer until clear visible growth is observed). Ideally, the culture tubes should fit into a portable spectrophotometer. In this way, the overnight grown culture can be easily adjusted to a standardized cell density by dilution with sterile ISB.
- **Two control cultures (*E. coli* LMG 8223 and ASIARESIST strain C256-V-OM-A-4-O-1) should be included during each series of MIC determinations.**
- Prepare a bottle with the necessary volume (see §.II) of sterile mQ (or double-distilled) water.

### II. Preparation and inoculation of the dilution series (DAY 3)

- The optical density of the overnight culture of the strain is determined spectrophotometrically at 590 nm and is standardized at  $0.1 \pm 0.02$  (i.e. app.  $10^8$  CFU/mL) by diluting with sterile ISB.
- For each batch of 10 strains (i.e. 9 test isolates and the reference strain), two 50 mL sterile stock solutions of chloramphenicol (i.e. stock solution 1 of 1024 ppm and stock solution 2 of 256 ppm) should be prepared in the suitable solvent (see Materials). However, subsequent dilutions of these stock solutions can be made up in sterile water. The use of two stock solutions is recommended to minimize quantitative errors in the low-concentration range of the serial dilution series.

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- For each set of 10 strains, a dilution series of two-fold antibiotic concentrations (4-1024 ppm) is prepared in sterile tubes of 50 mL according to the scheme outlined in Table 1. In the first step, the antibiotic concentrations 512 and 256 ppm are prepared from stock solution 1 (1024 ppm) by adding equal volumes of sterile mQ. Antibiotic concentrations 128, 64, 32, 16, 8, and 4 ppm are obtained by making serial dilutions from stock solution 2 (256 ppm) in a second step. It is absolutely crucial to thoroughly mix every freshly prepared antibiotic dilution prior to using it to prepare the next dilution. It should be noted that each antibiotic dilution undergoes a final 1:2 dilution when the broth culture is added (Table 2). **If required, the tested MIC range can be extended with additional concentrations.**

**Table 1. Scheme for preparation of antibiotic dilution series (per 10 strains)**

Tube no	Concentration obtained	Volume of AB solution	Volume of sterile mQ
1	1024 ppm	25 mL (stock solution 1)	-
2	512 ppm	25 mL (1024 ppm)	25 mL
3	256 ppm	25 mL (512 ppm)	25 mL
4	128 ppm	25 mL (stock solution 2)	25 mL
5	64 ppm	25 mL (128 ppm)	25 mL
6	32 ppm	25 mL (64 ppm)	25 mL
7	16 ppm	25 mL (32 ppm)	25 mL
8	8 ppm	25 mL (16 ppm)	25 mL
9	4 ppm	25 mL (8 ppm)	25 mL

Stock solution 1 = 1024 ppm; stock solution 2 = 256 ppm.



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- Following the preparation of the serial antibiotic dilutions, 2 mL of freshly standardized broth culture of the strain is inoculated in each tube of the dilution series according to the scheme outlined in Table 2. In this regard, it is important to note that the standardized cultures should be processed within the hour after preparation. Dilutions and broth cultures should be well homogenized prior to mixing.

**Table 2. Scheme for preparation of culture dilution series (per strain)**

MIC no	Final concentration	Volume of AB solution	Volume of broth culture
1	512 ppm	2 mL (stock solution 1; tube 1)	2 mL
2	256 ppm	2 mL (512 ppm; tube 2)	2 mL
3	128 ppm	2 mL (256 ppm, tube 3)	2 mL
4	64 ppm	2 mL (128 ppm; tube 4)	2 mL
5	32 ppm	2 mL (64 ppm; tube 5)	2 mL
6	16 ppm	2 mL (32 ppm; tube 6)	2 mL
7	8 ppm	2 mL (16 ppm; tube 7)	2 mL
8	4 ppm	2 mL (8 ppm; tube 8)	2 mL
9	2 ppm	2 mL (4 ppm; tube 9)	2 mL

- For each batch of MIC determinations, a blanc tube (i.e. 2 mL non-inoculated ISB mixed with 2 mL water) should be included. In addition, a positive control should be included for each strain. The positive control is made up by mixing 2 mL adjusted broth culture with 2 mL sterile mQ (or double-distilled) water.
- All MIC tubes (concentration range 2-512 ppm) and control tubes of the test isolates and the control strain as well as the blanc are incubated aerobically at 28°C for 24h.
- Each isolate should also be checked for purity by plating a drop of the adjusted culture onto ISA medium, and this plate should be incubated under the same conditions as the MIC test itself.

### III. Reading of the MIC (DAY 4)



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- The purity of the broth culture is checked on ISA on the basis of uniform colonial morphology. If contamination is noted than all data generated from the involved strain should be rejected.
- Following a 24 h incubation (or longer until clear visual growth can be determined in the positive control tubes), growth is determined visually among the different tubes of the serial dilution by comparing with the positive control and with the blanc. Any series where discontinuity in growth is observed (e.g. growth in tubes 5 and 7 but not in tube 6) should be discarded. The end-point is defined as the lowest antibiotic concentration for which there is no visual growth. This concentration should be reported as the MIC of that antibiotic for that particular strain. If trailing end-points are observed, this should be reported as a remark and a 80% reduction in growth should be reported as end-point.
- Both the NCCLS and BSAC guidelines consider that a MIC of  $\geq 16$ ppm indicates resistance to chloramphenicol.

#### IV. Harmonization of method between collaborating laboratories

##### (IGNORE AFTER COMPLETION OF HARMONIZATION)

- In order to correlate MIC data determined in different laboratories, it is necessary to harmonize the inter-laboratory logistics at the start of each major survey. For this purpose, the results of MIC determination of chloramphenicol for a set of six reference strains should be circulated between laboratories. Two of these reference strains will subsequently be used as control strains to be included in each batch of MIC determinations. According to BSAC guidelines, *E. coli* strain LMG 8223 has an MIC value of 4 ppm for chloramphenicol. The MIC of strain C256-V-OM-A-4-O-1 was determined at  $\geq 256$  ppm using the agar dilution method, but also needs to be determined using the method described in this SOP.

#### C. REFERENCES

- Smith, P. and colleagues of the Working Group on Methods for Antimicrobial Agent Susceptibility Testing of Bacteria Associated with Fish Diseases. 2000. Towards a set of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. <http://www.nuigalway.ie/mic/eusus/>
- Antimicrobial susceptibility testing: BSAC working party report. J. Antimicrob. Chemother. 48, Supplement S1 (2001). Free download at [http://www.jac.oupjournals.org/content/vol48/suppl\\_1/](http://www.jac.oupjournals.org/content/vol48/suppl_1/)

### D. MATERIALS

- **Iso-Sensitest agar and broth (ISA and ISB) medium**  
# CM 473 and #CM471 (Oxoid, Basingstoke, UK)

*Media should be prepared with distilled or freshly deionised water according to the manufacturer's instructions. The pH is set at pH 7.4 ± 0.2. Poured ISA plates may be stored for up to 2 weeks in air-tight plastic bags at 2-8 °C. Immediately prior to inoculation ISA plates should be moist but free of droplets, which should not be present on either the agar surface nor on the petri dish lids. If necessary plates may be dried by incubation at 30-37 °C or in a laminar flow cabinet for a maximum of 30 min. A representative sample of each batch of ISB tubes or ISA plates should be examined for sterility by incubation at 28 °C for 72 h.*

- **Chloramphenicol (CHL) stock solutions (256 ad 1024 ppm)**  
*Preferably, all participating labs should use the same brand of chloramphenicol. In the current project, all partners have agreed to purchase CHL from Oxoid in the form of Selective Supplement vials containing 50 mg CHL each (# SR0078, Oxoid).*  
*The CHL stock solution needs to be prepared in 95% ethanol according to the manufacturer's instructions (<http://www.oxoid.com/uk/index.asp?mpage=iproductdetail&pre=SR0078&l=EN&x=>). Consequently, it is not necessary to filter-sterilize the solution as the chance that bacteria can survive in ethanol is negligible. The stock solutions should be stored in the refrigerator until preparation of the serial dilutions.*
- **Control strain LMG 8223 (= ATCC 25922 = NCTC 12241 = NCIMB 12210)**  
Fresh subculture the *E. coli* reference strain should be available on day 1.

- **Control strain C256-V-OM-A-4-O-1**

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Fresh subculture this control strain should be available on day 1.

- **Reference strain harmonization set (Ignore after completion of harmonization)**

1. ASIARESIST strain C256-V-OM-A-4-O-1 (reference strain for each batch)
2. *Escherichia coli* LMG 8223 (reference strain for each batch)
3. *Aeromonas hydrophila* LMG 2844
4. *Stenotrophomonas maltophilia* LMG 11098
5. *Acinetobacter junii* LMG 10577
6. *Salmonella enteritidis* LMG 10395

- **Sterile water**

milli Q (deionized)water or double-distilled water containing 0.85% NaCl