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SOP

Standard Operating Procedure

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Title:

ANTIBIOTIC SUSCEPTIBILITY TESTING OF AQUACULTURE-ASSOCIATED BACTERIA WITH THE DISC DIFFUSION METHOD

References:

See section C

Reviewed & approved by: all ASIARESIST partners

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ANTIBIOTIC SUSCEPTIBILITY TESTING OF AQUACULTURE-ASSOCIATED BACTERIA WITH THE DISC DIFFUSION METHOD

A. PURPOSE & PRINCIPLE

The purpose of this SOP is to describe the procedure for testing the antimicrobial susceptibility of bacterial isolates using the disc diffusion methodology *(i.e. antibiogram determination*). The method indicates susceptibility of the challenged organism to the tested antibiotic by a clear zone of inhibited growth around the impregnated filter paper discs. Upon contact with the agar surface, the antibiotic drug diffuses into the medium. The growth of the organism is inhibited until a critical concentration (comparable to the Minimal Inhibitory Concentration or 'MIC') is achieved. The diameter of the resulting zone is considered proportional to the degree of susceptibility and allows to categorise the organism into susceptible (S), intermediate (I), or resistant (R) when comparing with international guideline tables.

B. METHOD DESCRIPTION

***Important note*:** Susceptibility tests will be performed at 28°C in correspondence to the mean water temperature of the regions in South-east Asia.

I. Bacterial cultivation and material preparations (DAY 1)

- The organism to be tested should be cultivated on a suitable agar medium under optimal incubation conditions to obtain a fresh overnight grown culture. <u>As standard conditions, growth on Iso-Sensitest Agar (ISA; Oxoid) at 28 °C under aerobic atmosphere are recommended</u>. Streak out the pure culture on an ISA plate in a way that distinct colonies will be obtained. Along with the tested

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organisms, a reference culture (*E. coli* LMG 8223) should be included during each series of antibiogram determinations.

- Prepare the desired volume of Iso-Sensitest Agar medium (Oxoid) according to the manufacturer's instructions. Before pouring the agar medium, bottles should be cooled to 40-50 °C in a water bath. All plates are poured on a flat, horizontal surface each to an identical depth of 5 mm $\pm$ 1 mm (corresponding to 20 mL $\pm$ 1 mL of medium in 10 cm radius petri dishes).
- Prepare tubes containing 5 mL sterile physiological solution (PHY-SOL)

II. Inoculation of the antibiogram (DAY 2)

- Harvest a number of distinct colonies from the fresh grown plate culture to suspend in a tube containing PHY-SOL until turbidity (visually) corresponding to 1.0 McFarland standard is reached. It remains important to take more than one colony in order to obtain a representative sample.
- Using a micropipet, spot 100 uL of the standardized suspension on the surface of an ISA plate. Spread plate the suspension using a sterile glass triangle rod. Allow to dry the plates for max. 15 minutes. Longer drying times allow pre-incubation of the cells which should be avoided. Where the room temperature is more than 5 °C above or below the incubation temperature, plates must be incubated within 10 minutes of the application of the discs. Where room temperature lies within 5 °C of the incubation temperature, plates should be incubated as soon as possible after the application of the discs.
- Using an Oxoid disc dispenser containing 6 agreed-upon antibiotics or manually using sterile forceps, the discs are applied onto the agar surface. Discs must not be relocated once they have made contact with the agar surface. Incubate the plates at 28°C for 24 hours.

III. Reading of the antibiogram (DAY 3)

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- The diameter of the inhibition zones are measured to the nearest mm from the point of abrupt inhibition of growth (using a callipers or mm ruler). Where there is any doubt the point of 80% inhibition should be taken as the zero edge.
- If the plates are not sufficiently grown, read again after 48 h incubation. Plates on which the growth of the test strain produces isolated colonies (less than semi-confluent growth) should not be read. If zones of inhibition produced by adjacent discs overlap to the extent that two measurements at right angles cannot be made, the zones around these discs should not be recorded. Equally, zones demonstrating significant distortion from circular should not be reported.
- If the zones of inhibition produced on plates inoculated with control strain *E. coli* LMG 8223 are not within the tolerance limits set (to be determined after the validation study; see IV), then all data collected in that particular set must be rejected. After the validation study performed by all partners, it was decided that a maximum between-batch variation of 3 mm is allowed as a quality control for zones determined with the control strain.
- The measured zones are compared with the NCCLS standard guidelines listed in Table 2 of Annex 1.

IV. Harmonization of method between collaborating laboratories
(IGNORE AFTER COMPLETION OF HARMONIZATION)

- In order to correlate antibiogram data determined in different laboratories, it is necessary to harmonize the inter-laboratory logistics at the start of each major survey. For this purpose, a set of taxonomically well-defined strains should be circulated between laboratories. Upon subculturing, each participating laboratory should perform the antibiogram determination during three independent trials according to the SOP outlined above for the following antibiotic discs: tetracycline (30 ug), oxytetracycline (30 ug), streptomycin (25 ug), chloramphenicol (30 ug),

ampicillin (25 ug), and oxolinic acid (2 ug). Statistical analysis of the triplicate data will determine the intra- and inter-laboratory reproducibility level.

C. REFERENCES

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D. MATERIALS

- **Iso-sensitest agar (ISA) medium**
  # CM 471 (Oxoid, Basingstoke, UK)

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Media should be prepared with distilled or freshly deionised water. Poured plates may be stored for up to 2 weeks in air-tight plastic bags at 2-8 °C. Immediately prior to inoculation media 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 plates should be examined for sterility by incubation at 28 °C for 72 h.

- **Antibiotic discs (*representing agents historically or currently used in aquaculture*)**
  from Oxoid (see catalogue at www.oxoid.com):
  1. tetracycline (30 ug, product code CT0054)[a]
  2. ampicillin (10 ug, product code CT0003)[b]
  3. chloramphenicol (30 ug, product code CT0013)
  4. nitrofurantoin (300 ug, product code CT0036)
  5. norfloxacin (10 ug, product code CT0434)
  6. trimethoprim/sulfamethoxazole 1:19 (Co-trimoxazole) (1.25/23.75 ug, product code CT0052) [c]

  [a]Tetracycline is tested as the class representative for chlortetracycline and oxytetracycline.
  [b]The results of ampicillin susceptibility tests may be used to predict susceptibility to amoxicillin and hetacillin.
  [c]This disc represents the potentiated sulphonamides and is used to test susceptibility to trimethoprim/sulfadiazine and ormethoprim/sulfadimethoxine.

  Discs in their original manufacturers packaging should be stored at refrigeration temperature. Once opened, a package should be stored in a tightly sealed container with a desiccant. If a commercial disc dispenser is employed, discs may be stored within the dispenser provided that the whole apparatus can be sealed and stored with a desiccant at refrigeration temperature. Discs must be disposed of when they have reached the manufacturer's expiry data.

- **Reference strain LMG 8223 (= ATCC 25922 = NCIMB 12210)**
  Fresh subculture the *E. coli* reference strain should be available on day 2

- **Reference strain harmonization set (<u>Ignore after completion of harmonization</u>)**

  Escherichia coli LMG 8223 (reference strain for each batch)

  Aeromonas hydrophila LMG 2844

  Stenotrophomonas maltophilia LMG 11098

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Vibrio harveyi LMG 4044

Acinetobacter junii LMG 10577

Salmonella enteritidis LMG 10395

- **Disc Dispenser**
  Oxoid

- **McFarland Standard**
  Series 0.5-1-2-3-4-5 McF
  bioMérieux (Marcy l'etoile, France)

- **Physiological solution (PHY-SOL)**
  milli Q (deionized)water or double-distilled water containing 0.85% NaCl

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ANNEX 1

Interpretive zone diameters in Table 1 are derived from NCCLS (*National Committee for Clinical Laboratory Standards*; USA) document M2-A5 (Performance standards for antimicrobial disk susceptibility tests) and from the BSAC (*British Society for Antimicrobial Chemotherapy*; UK) paper describing a standardized disc susceptibility testing method (JAC 48:S1, 2001). The first values in Table 1 are those recommended by NCCLS and were determined using Mueller-Hinton (M-H) medium for fast-growing organisms incubated at 37°C. For all discs relevant to this project, NCCLS has proposed zone diameters. The second values (between brackets) in Table 1 are those of BSAC and were determined for Enterobacteriaceae and acinetobacters on Iso-Sensitest Agar (ISA) grown at 35-37°C. Strikingly, the BSAC guidelines often do not list values for the I (intermediate) group as is the case with the NCCLS guidelines. In the framework of the EU-ASIARESIST project, the current BSAC guidelines (version July 2001) have two specific shortcomings: (i) a number of antibiotics relevant in aquaculture are not listed such as nitrofurantoin and norfloxacin, and (ii) except for ampicillin, the interpretive zones listed for the remaining test agents (i.e. tetracycline, chloramphenicol, and co-trimoxazole) are considered 'tentative' by the authors of the JAC paper. This means that for these antibiotics, few BSAC data are available or are coming from a single laboratory, and that in conclusion these guidelines should/can not be taken too strict. This can be clearly noted when comparing the NCCLS and BSAC guidelines for tetracycline. On the other hand, the zones for ampicillin show a relatively good correlation between NCCLS and BSAC interpretations.

The above mentioned findings clearly illustrate the long-time 'dilemma' in choosing between M-H and ISA medium when performing disc diffusion susceptibility testing (Gould, 2000; Koeth *et al*., 2000), in particular on environmental bacteria. On the one hand, M-H agar has the longest history of use and consequently most diffusion data have been generated on this medium making it possible to construct representative interpretative tables. A drawback of M-H agar use is its suspected antagonistic activity especially when testing tetracyclines, trimethoprim, and sulphonamides, three groups of agents

of specific importance to this project. On the other hand, ISA medium was designed to minimize these suspected antagonistic effects through a stabililized mineral content. However, because of its relatively short history of usage there are insufficient data available for interpretative reading of ISA antibiograms. Taken together, the values listed in Table 1 leaves two options: (i) as only NCCLS guidelines provide data for all discs relevant to the project, we may consider to perform disc diffusion susceptibility testing on Mueller-Hinton agar rather than on ISA or (ii) all partners continue using the ISA medium (hereby ensuring a minimum of antagonistic effects) for disc diffusion susceptibility testing whereas for interpretation NCCLS guidelines should be used since BSAC guidelines are only tentative for most discs. Clearly, workers should try to find a 'best-of-both-worlds' solution in this matter. UPON AGREEMENT AMONG ALL PROJECT PARTNERS, THE SECOND OPTION WILL BE CONSIDERED FOR THE EU-ASIARESIST PROJECT, I.E. THE SOP-ASIARESIST-ABR WILL REMAIN AS IT IS AND NCCLS GUIDELINES (SEE TABLE 2) WILL BE USED FOR INTERPRETATION.

Table 1. NCCLS and BSAC guidelines for proposed discs

DISC	Resistant	Intermediate	Susceptible
Tetracycline (30)	≤14 (≤33)	15-18	≥19 (≥34)
Ampicillin (10)	≤13 (≤17)	14-16	≥17 (≥18)
Chloramphenicol (30)	≤12 (≤20)	13-17	≥18 (≥21)
Nitrofurantoin (300)	≤14 (nd)	15-16	≥17 (nd)
Norfloxacin (10)	≤12 (nd)	13-16	≥17 (nd)
Trimethoprim/sulfamethoxazole (1.25/23.75)	≤10 (≤15)	11-15	≥16 (≥16)

Table 2. NCCLS guidelines used as consensus for reading of ASIARESIST antibiograms

DISC	Resistant	Intermediate	Susceptible
Tetracycline (30)	≤14	15-18	≥19

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Ampicillin (10)	≤13	14-16	≥17
Chloramphenicol (30)	≤12	13-17	≥18
Nitrofurantoin (300)	≤14	15-16	≥17
Norfloxacin (10)	≤12	13-16	≥17
Trimethoprim/sulfamethoxazole (1.25/23.75)	≤10	11-15	≥16