D-JRP15-FED-AMR-WP2.1

Laboratory Operating Procedure (LOP)
Protocol for Microbiology

Version 1
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A. Description

The purpose of this procedure is to provide a harmonizing framework in the microbiology laboratory, for the isolation, detection and identification of specific bacterial isolates from different samples collected (**Appendix 1**), such as faeces (from pigs, wild animals and farmers), manure, soil, water, crops and feed. This procedure applies to bacterial isolates that may have human, veterinary, zoonotic or environmental bacteria, in such samples, focusing on the following genera/species:

- *Escherichia coli* (ideally ESBL-/AmpC-producing *E. coli* and/or positive for *mcr*-variants)
- *Klebsiella pneumoniae* (ideally ESBL-/AmpC-producing *K. pneumoniae* and/or positive for *mcr*-variants)
- *Salmonella* spp
- *Staphylococcus aureus* (ideally methicillin-resistant *S. aureus,* MRSA)
- *Enterococcus faecium/faecalis* (ideally vancomycin-resistant *E. faecium/E. faecalis*, VRE)

B. Equipment and material

- Refrigerator, capable of operating at 5ºC ± 3ºC
- Freezer, capable of operating at - 20ºC, or - 80ºC
- Incubators, capable of operating at several temperatures
- Water baths, capable of operating at several temperatures
- Sterile flasks, test tubes or screw-cap bottles, of appropriate capacities
- Sterile screw-capped 1.5 mL conical tubes (Eppendorf, or similar)
- Sterile cryotubes
- Sterile scoop or spatula
- Bunsen burner
- Sterile loops, of approximate diameter, 3 mm (10 µl volume), and of 1 µl volume
- Forceps
- Sterile graduated pipettes or automatic pipettes, of nominal capacities of 25 ml, 10 ml, 1 ml, and 0.1 ml
- Pipettors with filtered sterile tips
- Sterile 90 mm Petri dishes
- Selective, nutrient, and pre-enrichment medium media
- Reference cultures for each genus (e.g. NCTC or ATCC)
- Bacterial identification tests (e.g. automated *biochemical* tests: Vitek; others: Maldi-Tof, PCR of 16S gene)
- Filtration system (Milipore)
- 0.45 µm and 0.2 µm cellulose nitrate sterile membrane filters (Whatman)
- pH Metter and/or pH Test Strip

C. Procedure

1. General conditions

Testing shall begin on **the day of receipt of samples**; the first working day should allow the method to be completed in accordance with the procedure.

If testing does not begin on the day of receipt, the samples should be stored accordingly with the nature of the sample, in conditions which will preserve its integrity (see Laboratory Operating Procedure (LOP) "sampling"). However, if samples have not been stored appropriately (0º to 5ºC) under transportation or storage shall be discarded.

All **microbiological media** may be: commercially ready-to-use, or prepared according to the manufacturer's instructions, or made in the laboratory by components (**Appendix 2**).

If refrigeration is necessary, samples should be removed from the refrigerator and stored at **room temperature** for a minimum of 1h prior to the start of the procedure.

To avoid damage to microorganisms by sudden changes in temperature, the temperature of all medium shall be approximately the same as the laboratory ambient temperature.

Samples must be **well mixed** using a sterile palate knife, spoon or stirring rod before subsampling, according to the nature of sample.

Do not homogenize **hard or dry products** in a rotary homogenizer for more than 2.5 min at one time to avoid an excessive rise in temperature. For some hard and dry products, it may be necessary to mince the laboratory sample, which should not be performed for more than 1 min, to avoid an excessive rise in temperature (see Laboratory Operating Procedure (LOP) "sampling").

For liquid and non-viscous products, before taking the test portion, the laboratory sample should be shaken by hand (e.g. 25 times through an arc of 25 cm) or by mechanical means in order to ensure that the microorganisms are uniformly distributed.

However, if the matrix contains many **particles** that may interfere with the detection of colonies, or if the level of bacteria is very low, this principle cannot be used without first separating the target microorganisms from the matrix (for example by filtration or immunoseparation).

For **acidic products** it is important when preparing a suspension that the pH is brought back to near neutrality (pH 7.0 ± 0.5). The use of buffered peptone water is sufficient for most products with pH greater than or equal to 4.5. More acidic products (greater than or equal to pH 3.5) may be brought back to the required pH using double-strength buffered peptone water, but the pH of such products should be checked when these are tested for the first time to ensure the required range is achieved.

The time between the end of the preparation of the initial suspension and the moment when the inoculum comes into contact with the final culture medium shall **not exceed 45 min**.

Positive and negative controls must be run on each day that testing is initiated.

2. Test portion

Aseptically weigh or measure the test portion, to a tolerance of ±5 %, into a sterile container or plastic bag. A mass of *m* g or a volume of *V* ml representative of the laboratory sample shall be used.

For mass *(m* g)***:***

- 0.5 g ± 0.1 g of soil or feces
- 1 g ± 0.1 g solid manure
- 1 g ± 0.1 g solid feed
- 10 g of crops' root
- 10 g of crops' stalk (caulosphere)
- 10 g of crops' ears (carposphere)
- 25 g of crops' leaves (phylloplane)

For volume *(V* ml)***:***

- 5 ml for water
- 5 ml for ground water/drinking water
- 5 ml for drainage water
- 5 ml for wastewater
- 5 ml for liquid manure
- 5 ml for liquid feed

3. <u>Prepare the initial suspension (non-selective pre-enrichment)</u>

- For preparation of the initial suspension use as diluent the pre-enrichment medium: buffered peptone water (BPW) (**Appendix 1**). Pre-warm the BPW to room temperature before use.
- An amount of test portion (*mass* or *volume*) is added to a quantity of BPW (*mass* or *volume*) in appropriate sterile tubes/beakers with lids to yield a **ten-fold dilution**. For this, according to the type of sample:
    - 0.5 g test portion of soil or feces is mixed with 4.5 ml of BPW
    - 1 g test portion of solid manure is mixed with 9 ml of BPW
    - 1 g test portion of solid feed is mixed with 9 ml of BPW
    - 10 g test portion of crops' roots is mixed with 90 ml of BPW
    - 10 g test portion of crops' stalks is mixed with 90 ml of BPW
    - 10 g test portion of crops' ears is mixed with 90 ml of BPW
    - 25 g test portion of crops' leaves is mixed with 225 ml of BPW
    - 5 ml of river water are diluted in 45 ml of BPW
    - 5 ml of ground water/drinking water are diluted in 45 ml of BPW
    - 5 ml of drainage water are diluted in 45 ml of BPW
    - 5 ml of wastewater are diluted in 45 ml of BPW
    - 5 ml of liquid manure are diluted in 45 ml of BPW
    - 5 ml of liquid feed are diluted in 45 ml of BPW
- Incubate the tubes (pre-enrichment culture) at 37ºC ± 1ºC for 4-6 h.
- This initial suspension of non-selective pre-enriched culture will be used for (see **Appendix 1**):
    - Isolation of *Escherichia coli* (see 4.)
    - Isolation of *Klebsiella pneumoniae* (see 5.)
    - Isolation of *Salmonella* spp. (see 6.)
    - Isolation of *Staphylococcus aureus* and MRSA (see 7.)
    - Isolation of *Enterococcus faecium* and *Enterococcus faecalis* (see 8.)

4. Isolation of *Escherichia coli* (ESBL-/AmpC-producing and/or positive for *mcr*-variants)

4a. Plating out on selective solid media for isolation of presumptive ESBL-/AmpC-producing *E. coli*

After mixing gently the incubated pre-enrichment culture in BPW (**3.**):

- Subculture by streaking one loopful (10 µl loop) over the surface of the MacConkey agar No.3 containing 1 mg/L of cefotaxime (CTX) (**Appendix 2**). To thin out the bacterial culture, from the first streak, make further two streaks using either the same loop or a 1 µl loop to ensure growth of single colonies. Incubate the plates at 44℃ ± 0.5℃ for 18-22 h.
- Based on colony morphology [presumptive *E. coli* colonies will usually be red/purple on the MacConkey agar plates containing 1 mg/L CTX] (Figure 1_Appendix 1).
- Sub-culture individual colonies onto MacConkey agar containing 1 mg/L CTX to maintain the selective pressure. Several colonies CTX-resistant should be individually subcultured.
- Incubate at 37℃ ± 1℃ for 18-22 h.
- Use ATCC or other control *Enterobacteriaceae* strains to control all medium.

4b. Identification of CTX-resistant *E. coli*

- Colonies of presumptive CTX-resistant *E. coli* are subcultured in Nutrient agar and their identity is confirmed by means of commercial bacterial identification test or apparatus (Vitek, Maldi-Tof, PCR of 16S gene, other).
- In case the first subculture is not identified as CTX-resistant *E. coli*, a second and eventually a third subculture (4a.) should be tested to have 4 CTX-resistant *E. coli* strains for following tests (Repeat Hypothesis ①_Figure 1_Appendix 1).
- If CTX-resistant *E. coli* strains are not detected, but only CTX-susceptible, 4 of these susceptible strains should be then retained for following tests (Hypothesis ②_Figure 1_Appendix 1).

4c. Confirmation of CTX-resistant *E. coli*

- Streak the selected colonies onto the surface of a pre-dried non-selective agar medium — Nutrient agar — in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates between 34ºC and 38ºC for 24 h ± 3 h. Use ATCC or other control Enterobacteriaceae strains to also control this medium.
- Alternatively, if well-isolated colonies (from a pure culture) are available on the selective plating media (4b.), the confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.
- Retain at least 4 colonies of pure culture [ideally CTX-resistant *E. coli* strains (Hypothesis ①_Figure 1_Appendix 1), but if at all impossible, then CTX-susceptible strains (Hypothesis ②_Figure 1_Appendix 1)] for further analysis (e.g. MICs; WGS), and freeze at - 20ºC or - 80ºC.

4d. In parallel to 4a., plating out on selective solid media, for isolation and identification of putative *mcr*-variant positive *E. coli* strains

After mixing gently the incubated pre-enrichment culture in BPW (**3.**):

- Subculture by streaking one loopful (10 µl loop) over the surface of the MacConkey agar containing 0.5 mg/L of colistin (COL) (**Appendix 2**). To thin out the bacterial culture, from the first streak, make further two streaks using either the same loop or a 1 µl loop to ensure growth of single colonies. Incubate the plates at 44℃ ± 0.5℃ for 18-22 h.
- Follow the method as it is described in item 4a., 4b. and 4c. (for CTX).
- At the end, select and retain at least 4 colonies of pure culture (ideally COL-resistant *E. coli* strains, but if at all impossible susceptible-COL strains) for further analysis (e.g. MICs; WGS), and freeze at -20ºC or - 80ºC.

5. Isolation of *Klebsiella pneumoniae* (ESBL-/AmpC-producing and/or positive for *mcr*-variants)

5a. Plating out on selective solid media for isolation of presumptive ESBL-/AmpC-producing *K. pneumoniae*

After mixing gently the incubated pre-enrichment culture in BPW (**3.**), follow the method as for *E. coli*, using also MacConkey agar No.3 or *Klebsiella* ChromoSelect Selective Agar containing 1 mg/L of cefotaxime (CTX) (4a.).

- Use ATCC or other control Enterobacteriaceae strains to control all medium.

5b. Identification and confirmation of CTX-resistant *K. pneumoniae*

Follow the method as for *E. coli* (4b. and 4c.).

5c. In parallel with 5a., plating out on selective solid media, for isolation and identification of putative *mcr*-variant positive *K. pneumoniae* strains

After mixing gently the incubated pre-enrichment culture in BPW (**3.**), follow the method as in 4d. using MacConkey agar No.3 or *Klebsiella* ChromoSelect Selective Agar.

6. Isolation of *Salmonella* spp

6.a. Selective enrichment for *Salmonella* spp.

After mixing gently the incubated pre-enrichment culture in BPW (**3.**):

- Inoculate 100 µl of pre-enrichment culture (obtained in 3.) in Rappaport-Vassiliadis broth with soya (RVS broth) or Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn broth) (Figure 2_Appendix 1).
- Incubate at 41.5ºC ± 1ºC for 24 h ± 3 h (RVS broth), or at 37ºC ± 1ºC for 24 h ± 3 h (MKTTn broth).
- For some products, it may be necessary to incubate the selective enrichment medium for an additional 24 h.
- Use ATCC or other control of *Salmonella* Typhimurium and/or *Salmonella* Enteritidis strains.

6b. Plating out on selective solid media for *Salmonella* spp.

From the culture obtained in the RVS broth or MKTTn broth (6a.):

- Inoculate by means of a 10 µl loop, the surface of a Xylose Lysine Deoxycholate agar (XLD) plate (selective solid media) so that well-isolated colonies will be obtained.
- XLD agar is incubated at 37ºC and examined after 24 h ± 3 h.
- Use ATCC or other control of *Salmonella* Typhimurium and/or *Salmonella* Enteritidis strains.

6c. Confirmation of *Salmonella* spp.

- Typical colonies of *Salmonella* on XLD agar (6b.) have a black centre and a lightly transparent zone of reddish color due to the color change of the indicator. *Salmonella* H_2S-negative variants grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening. Colonies of *E. coli* are yellow or yellow to reddish-yellow (Figure 2_Appendix 1).
- Colonies of presumptive *Salmonella* are subcultured in Nutrient agar and their identity is confirmed by means of bacterial identification test or apparatus (Vitek, Maldi-Tof, PCR of 16S gene, other).
- If negative, select up to 4 more suspect colonies ensuring that these colonies are subcultured from different selective enrichment/isolation medium combinations showing suspect growth.
- Streak the selected colonies onto the surface of a pre-dried non-selective agar medium — Nutrient agar — in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates between 34ºC and 38ºC for 24 h ± 3 h. Use ATCC or other control *Enterobacteriaceae* strains to control this medium.
- Alternatively, if well-isolated colonies (from a pure culture) are available on the selective plating media, the confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.
- Retain at least 4 colonies of pure culture for further analysis (e.g. MICs; WGS), and freeze at - 20ºC or - 80ºC.

7. <u>Isolation of MRSA</u>

7a. Selective enrichment for *S. aureus*

After mixing gently the incubated pre-enrichment culture in BPW (**3.**):

- Inoculate 2 ml on thioglycollate enrichment broth (Figure 3_Appendix 1).
- Incubate at 37ºC for 16-24 h.

7b. Plating out on two selective solid media (for MRSA)

- Inoculate by means of a 10 µl loop (from 7a.), the surface of MRSA selective agar CHROMagar MRSA (MAST Diagnostica, Reinfeld, Germany) as an *initial screening* step, and well-isolated colonies will be obtained.
- Incubate for 40–48 h at 37 ℃.

- Suspicious colonies are mauve-colored colonies (Figure 3_Appendix 1.
- These colonies (mauve-colored) should be selected for growing on another MRSA-selective chromogenic agar, chromID MRSA (bioMérieux), *to confirm* (green colonies) *or contradict the result of the screening* (CHROMagar MRSA-based) (Figure 3_Appendix 1).
- Incubate for 40–48 h at 37 ℃.
- Select the green colonies.

7c. Confirmation of *S. aureus*

- Strains that were presumptively MRSA by ChromID MRSA testing, should be confirmed to be *S. aureus* by means of bacterial identification test or apparatus (Vitek, Maldi-Tof, PCR of 16S gene, other).
- Confirm if *S. aureus* colonies are MRSA: use Vitek, perform a PCR (according to the EURL-AR "Protocol for PCR Amplification of *mecA, mecC, spa* and *pvl*", available at https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/279_pcr-spa-pvl-meca-mecc-sept12.pdf), or other method.
- If negative, select up to 4 more suspect colonies ensuring that these colonies are subcultured from different selective enrichment/isolation medium combinations showing suspect growth.
- Streak the selected colonies onto the surface of a pre-dried non-selective agar medium — Nutrient agar — in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates between 34ºC and 38ºC for 24 h ± 3 h. Use ATCC or other control *S. aureus* strains to control this medium.
- Alternatively, if well-isolated colonies (from a pure culture) are available on the selective plating media, the confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.
- Retain at least 4 colonies of pure culture [ideally MRSA (Hypothesis ①_Figure 3_Appendix 1), but if at all impossible, then MSSA strains (Hypothesis ②_Figure 3_Appendix 1)] for further analysis (e.g. MICs; WGS), and freeze at - 20ºC or - 80ºC.

8. Isolation of VRE *(Enterococcus faecium* and *Enterococcus faecalis)*

8a. Plating out on *Enterococcus* selective solid media:

- After mixing gently the incubated pre-enrichment culture in BPW (**3.**):
- Subculture by streaking one loopful (10 µl loop) over the surface of ChromID VRE agar (BioMérieux) and incubate at 36º ± 2ºC for 24 ± 4 h.

8b. Isolation and identification of *Enterococcus faecium/Enterococcus faecalis* (VRE)

-Typical VRE colonies in ChromID VRE agar are (Figure 4_Appendix 1):

- *E. faecium* appears as violet colonies
- *E. faecalis* appears as blue-green colonies

8c. Confirmation of *Enterococcus*

Strains that were presumptive VRE *Enterococcus*, should be finally confirmed by means of commercial bacterial identification test or apparatus (Vitek, Maldi-Tof, PCR of 16S gene, other).

- If negative, select up to 4 more suspect colonies ensuring that these colonies are subcultured from different selective enrichment/isolation medium combinations showing suspect growth.
- Streak the selected colonies onto the surface of a pre-dried non-selective agar medium — Nutrient agar — in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates between 34ºC and 38ºC for 24 h ± 3 h. Use ATCC or other controls of *E. faecium and/or E. faecalis* strains to control the medium.
- Alternatively, if well-isolated colonies (from a pure culture) are available on the selective plating media, the confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.
- Retain at least 4 colonies of pure culture [ideally VRE (Hypothesis ①_Figure 4_Appendix 1), but if at all impossible, then VSE strains (Hypothesis ②_Figure 4_Appendix 1)] for further analysis (e.g. MICs; WGS), and freeze at - 20ºC or - 80ºC.

9. <u>Membrane filtration of water samples</u>

- Perform the filtration of a known volume of water sample through 0.45 µm cellulose nitrate sterile membrane, using a filtering system (Figure 1).

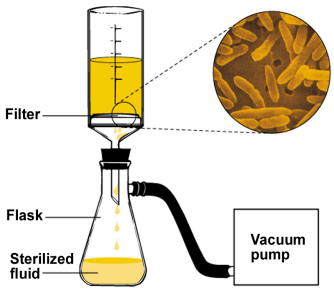

Figure 1. Filtration system

- Place the filter on the first solid medium indicated previously in item 4. to 8. (Figure 2).
- Follow the protocol for each of those bacteria.
- Examine the membranes and select the colonies that grew on the filter, with the respective morphology and color (Figure 2), for detection and identification of *E. coli* (see 4.) and *K. pneumoniae* (see 5.) (both ideally resistant to cefotaxime and/or to colistin), as well as of *Salmonella* spp. (see 6.), *S. aureus* (ideally MRSA) (see 7.), and *E. faecium* and *E. faecalis* (ideally VRE) (see 8.).

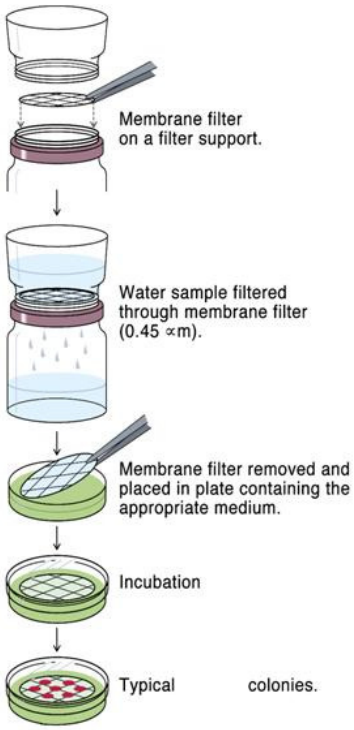

Figure 2. Bacterial culture and selection of typical colonies

10. Dilutions of samples *vs* the need to obtain isolated colonies in culture

There is no need to do dilutions of samples as the goal is not enumerating colonies.

To obtain isolated colonies is necessary, we must use two loops to scratch the same plate in order to obtain isolated colonies: scratching the first quarter of the plate with a loop of 10 µl, and line there once with a loop of 2 µl and scratching in the other three quarters of the plate.

11. Validation of selective and indicative agar plates

A Quality Control (QC) procedure to validate each type of medium used in the protocol (e.g. selective agar plates, and others) should be performed by using negative and positive control strains.

- Use ATCC or other control bacterial strains with phenotypic characteristics needed to control the medium (e.g. CTX-resistant *K. pneumoniae* strain and CTX-susceptible *K. pneumonia* strain to control the MacConkey agar plates containing 1 mg/L of cefotaxime).
- Negative control broth or plates are checked for sterility. If at any stage the negative control plate demonstrates growth the procedure is invalidated.

D. References:

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Appendix 1

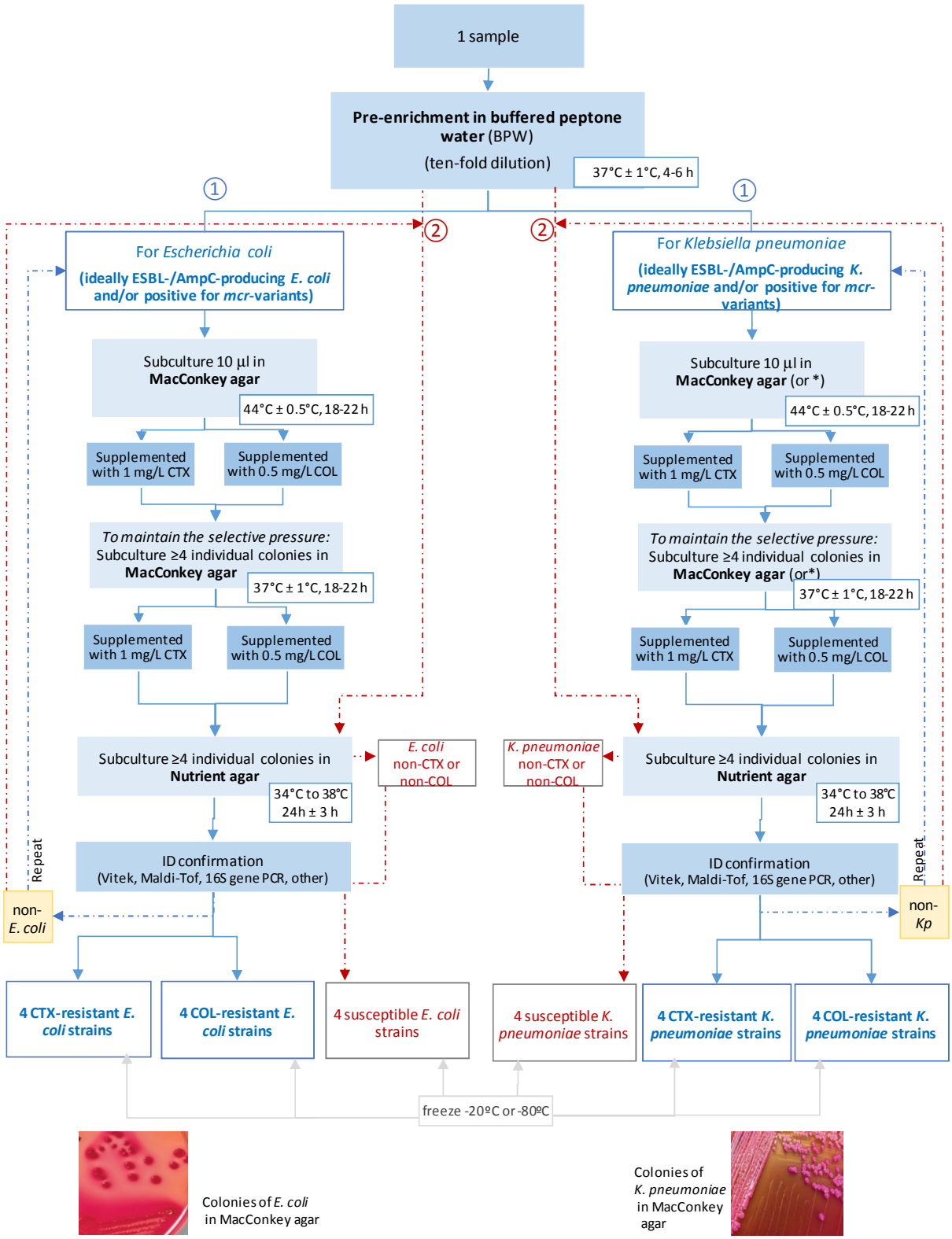

Fig 1. Isolation of (A) *E. coli* and (B) *K. pneumoniae* (ESBL-/AmpC-producing and/or positive for *mcr*- variants)

Hypothesis ① Main protocol for isolation of ESBL-/AmpC-producing and/or positive for *mcr*-variants *E.coli* and *K. pneumoniae* strains.
Hypothesis ② If CTX- or COL-resistant strains are not detected, subculture the BPW directly in anon-selective media (Nutrient agar).
* ***Klebsiella*** **ChromoSelect Selective Agar**

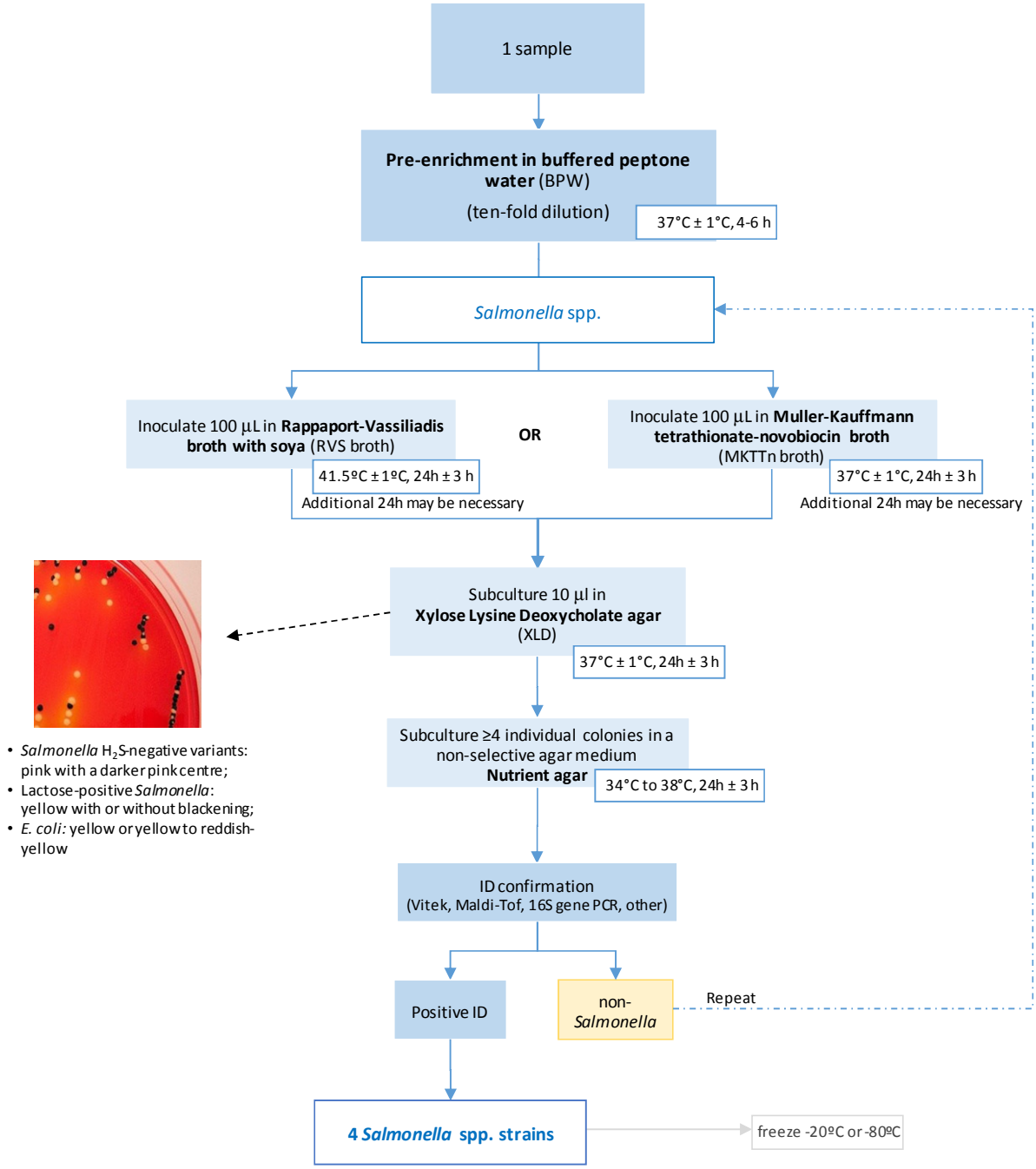

Fig 2. Isolation of *Salmonella* spp.

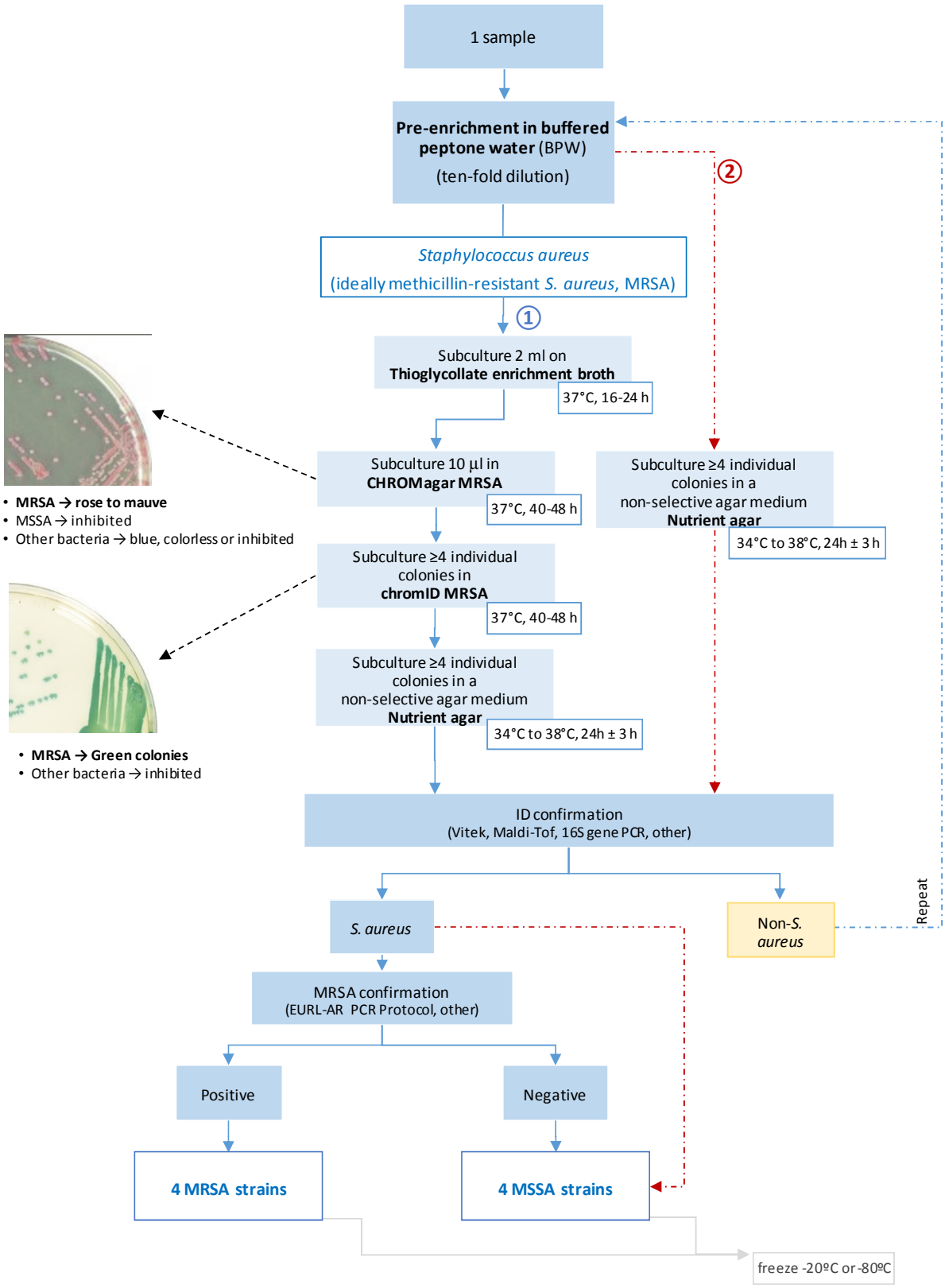

Fig 3. Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA).

Hypothesis ① Main protocol for isolation of MRSA strains.
Hypothesis ② If MRSA strains are not detected, subculture the pre-enrichment in buffered peptone water (BPW) directly in a non-selective media (Nutrient agar).

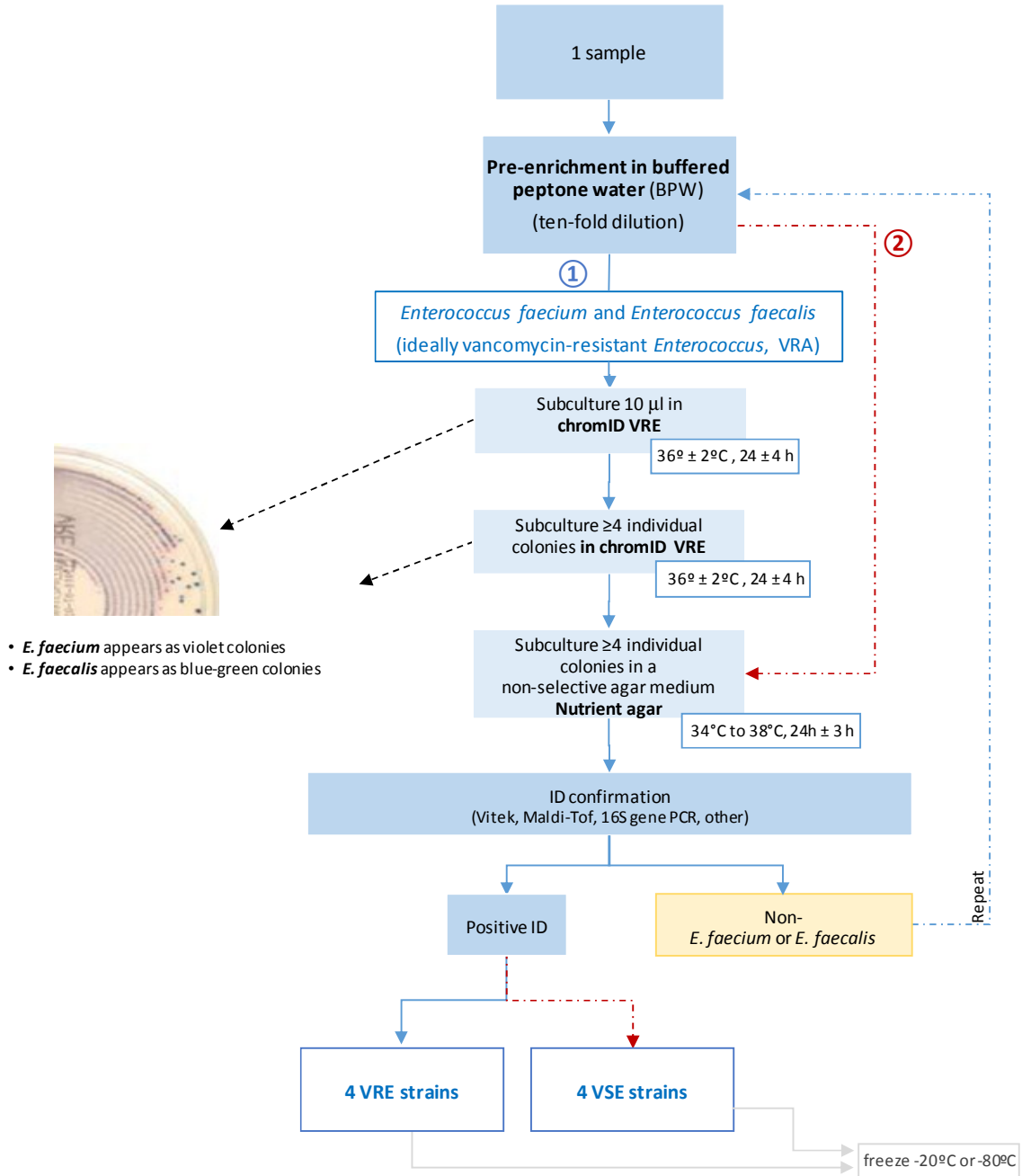

Fig 4. Isolation of VRE *(Enterococcus faecium and Enterococcus faecalis)*

Hypothesis ① Main protocol for isolation of VRE strains.
Hypothesis ② If VRE strains are not detected, subculture the pre-enrichment in buffered peptone water (BPW) directly in a non-selective media (Nutrient agar).

Appendix 2

The broth, agar medium and reagents are:

- purchased already made from several companies or
- dehydrated media performed in the laboratory according to the manufacturer's instructions [Note: the media should be prepared according to the manufacturer's instructions, if they differ from the description given here], or
- made in the laboratory by components, as follows:

Diluents for general use:

Buffered peptone water (BPW)

Composition:

Peptone[a]	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)[b‡]	9.0 g
Potassium dihydrogen phosphate (KH_2PO_4)[‡]	1.5 g
Water	1 000 ml

[a] For example, enzymatic digest of casein.

[b] If disodium hydrogen phosphate with a different water content is used, amend the mass of the ingredient accordingly. For example, in case of anhydrous disodium hydrogen phosphate (Na2HPO4), use 3.57 g.

Preparation:

Dissolve the components in the water in flasks, bottles or test tubes (6.4), by heating if necessary. Adjust the pH, if necessary, so that after sterilization, it is 7,0 ± 0,2 at 25 °C.

[‡] Buffer ingredients, see 5.2.3.

MacConkey No.3

Composition:

Peptone	20.0 g
Lactose	10.0 g
Bile salts No. 3	1.5 g
Sodium chloride	5.0
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
pH 7.1 +/- 0.2 (25°C)	

Preparation:

Suspend 51.5 g in 1 L of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Selective Supplement

Composition:

Cefotaxime[a] sodium salt stock solution prepared in bi-distilled water	1 mg/ml
Colistin	0.5 mg/ml

[a] It is important to take into account the potency of the drug to ensure that 1 mg/ml active compound is used. Aliquots of aqueous cefotaxime stock solution (concentration 1 mg/ml) can be stored at -20℃. [Example: If the manufacturer has stated a potency of 50%, 2 mg of antibiotic powder should be added to 1 mL of sterile dH2O (or the solvent routinely used) to reach a final concentration of 1 mg/mL of active compound.]

Nutrient agar (example of non-selective medium)

Composition:

Meat extract	3,0 g
Peptone	5,0 g
Sodium chloride (NaCl) (optional)	5,0 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar

Preparation:

Dissolve the components or the dehydrated complete medium in the water by heating, with frequent agitation.
Adjust the pH, if necessary, so that after sterilization, it is 7.0 ± 0.2 at 25ºC.
Transfer the culture medium into tubes or flasks of appropriate capacity.
Sterilize for 15 min in the autoclave set at 121ºC.

Preparation of nutrient agar plates:

Cool the medium to 47ºC to 50ºC in a water bath, mix, and pour into sterile Petri dishes. Allow to solidify. Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven set between 25ºC and 50ºC until the surface of the agar is dry.
Store the poured plates protected from drying, at 5ºC for up to four weeks.

***Klebsiella* ChromoSelect Selective Agar**

Composition:

bile salts mixture	1,5 g
Peptone special	12,0 g
Sodium chloride	5,0 g
Sodium lauryl sulfate	0,1 g
Yeast extract	7,0 g
chromogenic mixture	0,2 g
Agar	15 g
Water	1 000 ml
pH 7.1±0.2 (25 ℃)	

Preparation:

Dissolve the components or the dehydrated complete medium in the water by heating, to dissolve completely. Sterilize by autoclaving at 121℃ for 15 minutes.

Rappaport-Vassiliadis medium with soya (RVS broth)

Composition:

Solution A	1000 ml
Solution B	100 ml
Solution C	10 ml

Preparation:

Adjust the pH, if necessary, so that after sterilization it is 5.2 ± 0.2 at 20 °C to 25 °C.

Dispense the medium into tubes or flasks of suitable capacity to obtain the portions necessary for the test, e.g. 10 ml quantities dispensed into tubes.

Sterilize for 15 min in the autoclave set at 115 °C.

Store the complete medium in closed tubes or flasks at 5 °C for up to three months.

Solution A

Composition:

Enzymatic digest of soya	5.0 g
Sodium chloride	8.0 g
Potassium dihydrogen phosphate (KH2PO4)	1.4 g
Dipotassium hydrogen phosphate (K2HPO4)	0.2 g
Water	1 000 ml

Preparation:

Dissolve the components in the water by heating to about 70 °C, if necessary.

The solution shall be prepared on the day of preparation of the complete RVS medium.

Solution B

Composition:

Magnesium chloride hexahydrate (MgCl2·6H2O)	400 g
Water	1000 ml

Preparation:

Dissolve the magnesium chloride in the water.

As this salt is very hygroscopic, it is advisable to dissolve the entire contents of MgCl2·6H2O from a newly opened container according to the formula. For instance, 250 g of MgCl2·6H2O is added to 625 ml of water giving a solution of total volume of 788 ml and a mass concentration of about 31,7 g per 100 ml of MgCl2·6H2O.

The solution may be kept in a dark glass bottle with tight stopper at room temperature for at least two years.

Solution C

Composition:

Malachite green oxalate	0.4 g
Water	100 ml

Preparation:

Dissolve the malachite green oxalate in the water.

The solution may be kept in a dark glass bottle at room temperature for at least eight months.

Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth

Composition:

Base medium	1000 ml
Iodine-iodide solution	20 ml
Novobiocin solution	5 ml

Preparation:

Aseptically, add 5 ml of the novobiocin solution to 1 000 ml of base medium . Mix, then add 20 ml of the iodine-iodide solution. Mix well. The final concentration of novobiocin in the complete medium is 40 mg/l.

Dispense the medium aseptically into containers of suitable capacity to obtain the portions necessary for the test, e.g. 10 ml quantities dispensed into tubes. After preparation, the pH of complete MKTTn broth will be approximately 8.0. If the complete medium is not used immediately, store it in the dark at 5℃. The pH may drop during storage due to chemical reactions. Do not use the complete medium if the pH drops below 7.0.

Base medium

Composition:

Meat extract	4.3 g
Enzymatic digest of casein	8.6 g
Sodium chloride (NaCl)	2.6 g
Calcium carbonate (CaCO3)	38.7 g
Sodium thiosulfate pentahydrate (Na2S2O3·5H2O)	47.8 g
Ox bile for bacteriological use	4.78 g
Brilliant green	9.6 mg
Water	1 000 ml

Preparation:

Dissolve the components or the dehydrated complete medium in the water by heating, with frequent agitation, until the medium starts to boil. Avoid overheating.

Adjust the pH, if necessary, so that it is 8.0 ± 0.2 at 25 ℃.

Thoroughly mix the medium.

The base medium may be stored in closed flasks at 5 ℃ for up to three months.

Iodine-iodide solution

Composition:

Iodine	20.0 g
Potassium iodide (KI)	25.0 g
Water	100 ml

Preparation:

Completely dissolve the potassium iodide in 10 ml of water, then add the iodine and dilute to 100 ml with sterile water. Do not heat.

Store the prepared solution in a (tightly) closed container in the dark for up to one year.

Novobiocin solution

Composition:

Novobiocin sodium salt	0.04 g
Water	5 ml

Preparation:
Dissolve the novobiocin sodium salt in the water.
Sterilize by filtration through a filter with a pore size of 0.22 µm.
The solution may be stored for up to four weeks at 5 °C or in small portions (e.g. of 5 ml) at -20 °C for up to one year.

Xylose Lysine Deoxycholate agar (XLD agar)

Composition:

Yeast extract	3.0 g
Sodium chloride (NaCl)	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
L-Lysine hydrochloride	5.0 g
Sodium thiosulfate	6.8 mg
Iron(III) ammonium citrate	0.8 g
Phenol red	0.08 g
Sodium deoxycholate	1.0 g
Agar	9 g to 18 g[a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

Preparation:
Dissolve the components or the dehydrated complete medium in the water by heating, with frequent agitation, until the medium starts to boil. Avoid overheating.
Adjust the pH, if necessary, so that the final pH shall be 7.4 ± 0.2 at 25ºC.
Pour the base medium into tubes or flasks of appropriate capacity.

Preparation of the agar plates:
Cool the medium to 47ºC to 50ºC in a water bath, mix, and pour into sterile Petri dishes. Allow to solidify.
Store the poured plates protected from drying, at 5ºC for up to four weeks.
Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven set between 25ºC and 50ºC until the surface of the agar is dry.

Thioglycollate enrichment broth

Composition:

Pancreatic Digest of Casein	15.0 g
Dextrose	5.5 g
Yeast Extract	5.0 g
Sodium Chloride	2.5 g
Sodium Thioglycollate	0.5 g
L-Cystine	0.5 g
Resazurin	1 mg
Agar	0.75 g
Water	1 000 ml

Preparation:
Dissolve the components or the dehydrated complete medium in the water by heating, with frequent agitation, until the medium starts to boil. Avoid overheating.
Adjust the pH, if necessary, so that the final pH shall be 7.1 ± 0.3 at 25ºC.

CHROMagar MRSA

Composition:

Agar	15.0 g
Peptones and yeast extract	40.0 g
Salts	25.0 g
Chromogenic mix	2.5 g
Water	1 000 ml

Preparation:
Disperse slowly 82,5g of powder base in 1L of purified water. Stir until agar is well thickened. Autoclave at 110℃ during 5 min.

ChromID MRSA

Composition:

Plant and animal peptones (porcine or bovine) 20.1 g	
Tris	0.65 g
Chromogenic mixture of α-glucosidase and a combination with cefoxitin	0.4 g
Selective mixture	4.1 g
Agar	13.0 g
Water	1 000 ml

Preparation:
Disperse slowly the powders base in 1L of purified water. Stir until agar is well thickened. Autoclave at 110℃ during 5 min.
Adjust the pH, if necessary, so that the final pH shall be 7.3 at 25ºC.

ChromID VRE agar

Composition:

Casein and meat peptone (bovine and porcine)	18.0 g
Heart peptone (bovine or porcine)	3.0 g
Corn starch	1.0 g
Sodium chloride	6.0 g
Agar	15.0 g
Mixture of 2 chromogenic substrates and vancomycin (8 mg/l)	0.12 g
Selective mixture	52.3 m
Water	1 000 ml

Preparation:
Disperse slowly the powders base in 1L of purified water. Stir until agar is well thickened. Autoclave at 110℃ during 5 min.
Adjust the pH, if necessary, so that the final pH shall be 7.2 at 25ºC.