SOP for WP 6: Prevalence and behavior of multiresistant gram-negative bacteria

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1. Objective

The main objective of WP 6 is to determine i) the prevalence and behavior of multiresistant gram-negative bacteria, i.e. extended-spectrum-ß-lactamase or carbapenemase producing *Enterobacteriaceae, Acinetobacter* and *Pseudomonas* and ii) their transmission routes related to irrigation water.

This Standard Operation Procedure aims at the standardization of the consortium partners work within WP6. It should enable the partners to handle samples correctly and send the isolates to the LGL.

2. Sampling

i) surface water samples (e.g. river water), n=30 samples/ country
ii) wastewater effluents, n=10 samples/ country
iii) irrigation water, n=10 samples/ country

Please specify:

- type of water i) or ii)
- source (e.g. river, groundwater) for ii)
- sampling date
- water/ air temperature

In case of wastewater effluents (treated wastewater, outlet) additionally:

- after or before disinfection (e.g. UV)

Samples of water (generally 200ml) should be collected in sterile glass, polyethylene or similar sterile containers and transported in a cold box with ice packs. Samples should be analyzed as soon as possible (in any case less than 24 hours after collection, storage temperature: 2 to 8 °C).

3. Material

- MacConkey agar with supplementation of antibiotics 2,5mg/l (**Appendix 2**)
- Brilliance CRE Medium, Oxoid Limited, Hampshire, UK
- Sheep blood agar, Oxoid Limited, Hampshire, UK (**Appendix 2**)
- Cryovials to cryopreserve the isolates in your lab, max. 500/ country (Appendix 4)
- BBL Culture Swabs (to send the isolates to LGL as pure cultures), BD, USA, max. 500/ country (Appendix 5)
- (Three) sieve plates
- 0,45µm sterile filters
- 5ml or 10ml sterile pipettes
- Sterile tweezers
- Alcoholic tissues
- Gas burner
- NaCl solution (negative control)
- NaCl 0,9% (deionized water) for negative control
- Filtration unit (**Appendix 3**).

4. Method

The whole equipment must be sterilized before use.

Filtrate 50ml and 20ml of each water sample. Use sterile tweezers to place the two filters on selective agar MaCConkey with Cefotaxime (two plates). Afterwards filtrate 50ml and place the third filter on selective CRE agar in the same way. Avoid air bubbles between the filter and agar surface. Incubate the plates at 37°C for 24 hours under aerobic conditions.

If the water is very dirty please filtrate additionally 5ml for MCC and 20ml for CRE. A negative control with NaCl 0,9% (deionized water) solution should be performed at the beginning and at the end of the filtration of water samples.

Sterilize the filtration unit after each water sample with alcohol tissues and a gas burner.

After the incubation period, count all the colonies on the filters, and specify total plate count/ volume filtered. Record the colony morphology (**Appendix 1**). Subcultivate max. 10 pure colonies with different morphology (shape, colour etc.) of each water sample on sheep blood agar and incubate the plates at 37°C for 24 hours under aerobic condition. Perform the Gram stain to confirm that the colonies are Gram negative.

Proceed with all Gram negative Isolates.
Keep the oxidase positive isolates with a *Pseudomonas* like colony morphology and oxidase negative isolates with the other morphologies described in **Appendix 1.** Please ensure that the isolates are pure cultures. If necessary purify by a second streaking to single colonies on sheep blood agar plates before cryopreservation. Please cryopreserve all the isolates in cryovials at your laboratory (Appendix 4).

5. Expression of results

Count the colonies (different morphologies) on the filters and express the results in CFU per 100 ml, and report the absence as “not detected” in the volume examined for each sample.

6. Collection and Transport

	1. Package			2. Package		
	Spain	SGL	DTU	Spain	SGL	DTU
Sending Month	20-21 June 2016	4. April 2016	11-12 July 2016	4-5 July 2016	27-28 june 2016	5-6 September 2016

For each isolate please proceed as follows:

One day before sending date:
From each cryovial please transfer a glass bead with attached bacteria onto a sheep blood agar plate. Please incubate the plate at 37°C for 24 hours under aerobic condition.

On the sending date:
Please collect with the BBL swab the isolate from the plate and transfer it inside of BBL culture medium (Appendix 5). Please use the BBL culture swabs as transport system and send them to LGL at ambient temperature. Use only fresh and pure colonies from sheep blood agar plates for inoculation of culture swabs.

If possible, please send us all the isolates collected in the culture swabs in two packages. Two packages/ country via express were mentioned in the LGL calculation. If it is neccesary to send us the isolates in more than two packages, please contact us in advance!
Delivery address:
Bayer. Landesamt f. Gesundheit und Lebensmittelsicherheit
Hygiene-Labor GE1
Nadera Hanifi
Veterinaerstr. 2
85764 Oberschleissheim

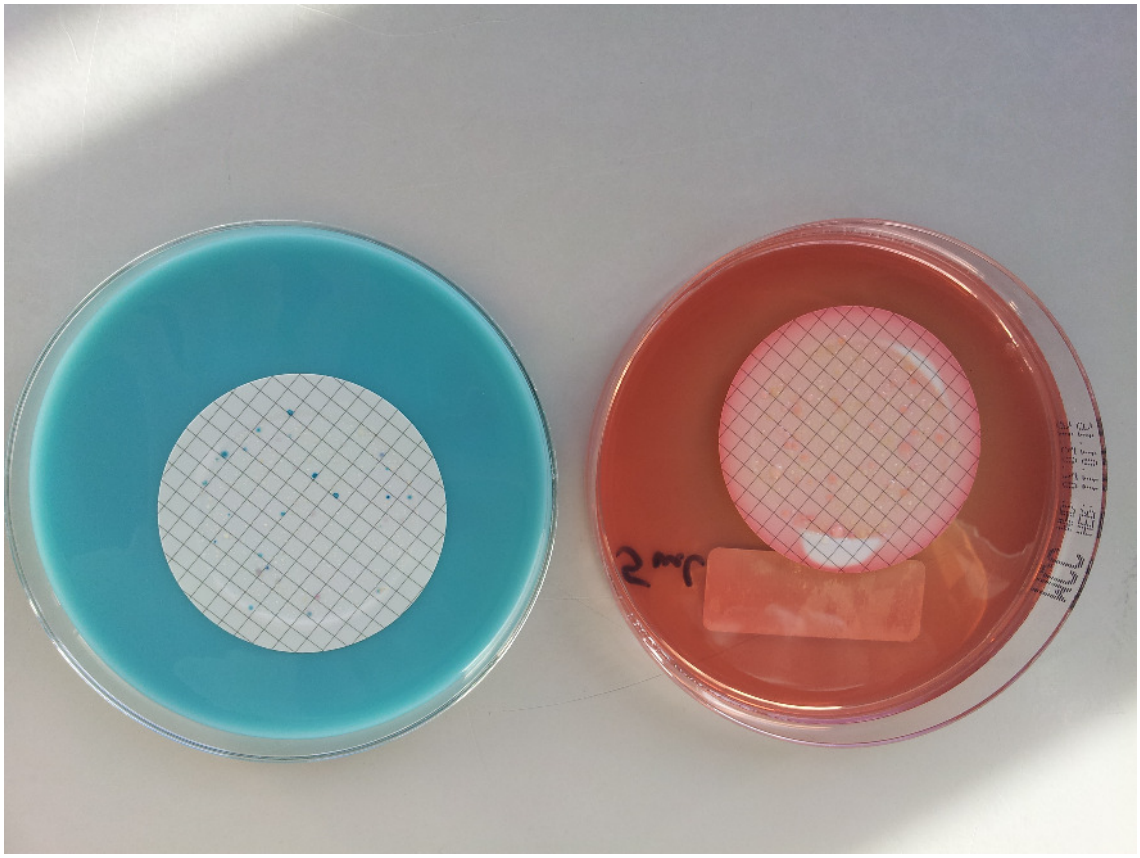
Tel.: ++49-9131-6808-5215

Appendix 1

Morphology of colonies frequently occuring within german water samples

Taxonomic group	MacConkey with supplement antibiotics	Brilliance CRE Medium, Oxoid	Oxidase Test
Pseudomonas	green (*aerugionsa*) or yellowish, brownish	purple	positive
Acinetobacer	yellow (*baumannii*) or white	small white or colourless	negative
KESC *(Klebsiella, Enterobacter, Serratia, Citrobacter)*	pink	blue	negative
Rahnella	pink	turqouise	negative
Escherichia coli	pink	pale pink	negative

Morphology of colonies frequently occuring within german water samples (left on CRE and right on MCC)



Appendix 2

Formulas of culture media

MacCONKEY Agar with supplement antibiotics (2,5 mg/l)

MacCONKEY Agar Oxoid CM 115
Formula (g / l): Peptone: 20.0; Lactose: 10.0; Bile salts No. 3: 31.5; Sodium chloride: 5.0; Neutral red: 0.03; Crystal Violett: 0.001; Agar: 15.0 (prepared according to the manufacturer's description)

Composition for 2 liters:
Suspend 103 g MacCONKEY Agar Oxoid (CM 115) in 2 liter of distilled water. Allow to swell for 10 min. Bring the suspension to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and add 10 ml antibiotic supplement.
Formula antibiotic supplement: 25 mg cefotaxime-sodium dissolved in 50 ml distilled water. Aliquots of 10 mL from these suspensions (sterile filtrate) can be stored at -20°C.

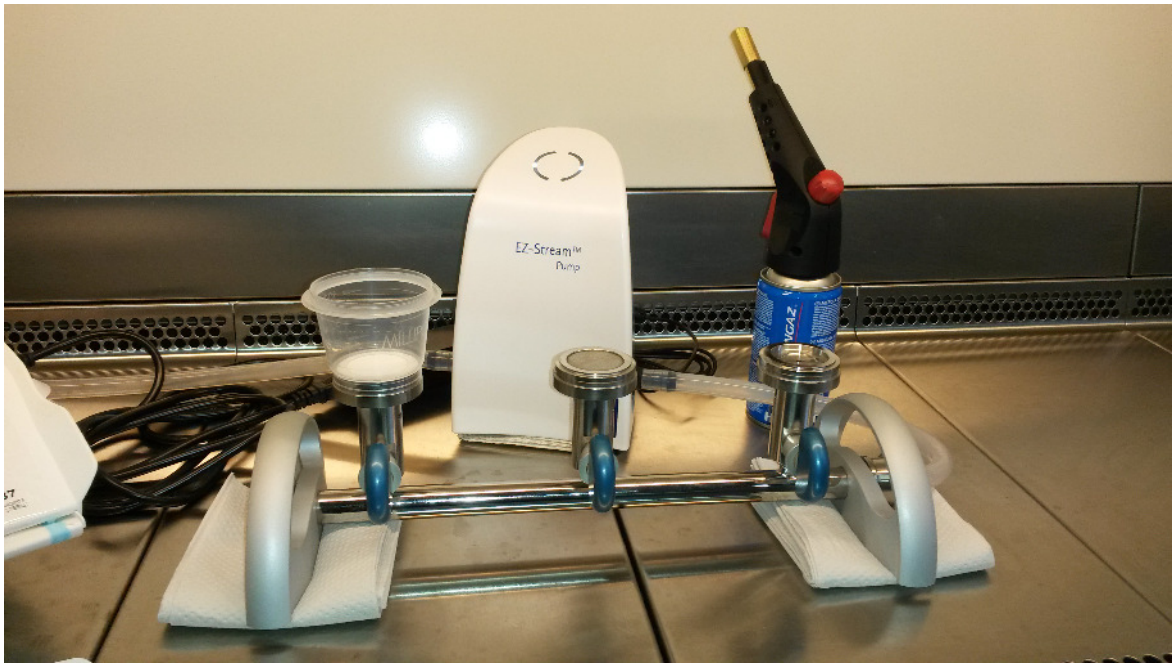
Sheep blood Agar:
Blood Agar Basis, Oxoid CM 55
Blood, Oxoid SR 0051E

Formula (g / l): meat extract ('Lab-Lemco'): 10.0; Peptone: 10.0; Sodium chloride: 5.0; Agar: 15.0 (prepared according to the manufacturer's description)

Composition for 5 liters:
Suspend 200 g blood Agar (Oxoid CM 55) in 5 liter of distilled water. Allow to swell for 10 min. Bring the suspension to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and add 10% Blood (Oxoid SR 0051E) (i.e. 500 ml Blood, Oxoid SR 0051E / 5 Liter Agar).

Appendix 3

Picture of the filtration unit at LGL



Appendix 4

Cryovials with beads

Appendix 5

BBL culture medium swab