DTU Food
National Food Institute

European Union Reference Laboratory Antimicrobial Resistance

Laboratory Protocol

Validation of selective MacConkey agar plates supplemented with 1 mg/L cefotaxime for monitoring of ESBL and AmpC producing *E. coli* in meat and animals

Final protocol, November 2015
Version 2

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HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
1	New document	-	21 Nov 2014	Authors
2	Version 2	Addition of information regarding 1) control strains for validation and 2) procedure when deviating results are observed	17 Nov 2015	Lina Cavaco, Susanne Karlsmose Pedersen
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Background

It is important for the harmonized monitoring of ESBL and AmpC producing *E. coli* to ensure the validity of the selective MacConkey agar plates supplemented with 1 mg/L cefotaxime. If these plates do not contain the correct concentration of the selective agent, false positive or false negative results can occur.

Based on Expert advice, the EURL AR has identified and validated a set of test strains with MIC values close to the selective concentration in the plates. The control set consists of two strains: a negative control and a positive control. Both these strains were shipped to the NRLs together with the EQAS trial for *Salmonella* and *Campylobacter* in October 2014. These were labeled as a negative and a positive control, respectively, for the ESBL/AmpC monitoring. The negative control will not grow on the MacConkey agar plates with 1 mg/L cefotaxime, while the positive control has the ability to grow on the MacConkey plates supplemented with this concentration of cefotaxime.

Please note that several different brands of MacConkey agar exist, and variation in their composition may influence the potency of the antimicrobial supplemented to the agar. It is therefore important to ensure that the chosen MacConkey agar (supplemented with the correct amount of cefotaxime) allow the positive test strain to grow according to the protocol given below, while it prevent growth of the negative control strain.

It is recommended that this protocol is carried out and validation of the plates is completed prior to performing the selective enrichment for ESBL and AmpC producing *E. coli* from meat and caecal samples. This is to avoid a situation where samples have been inoculated on plates that do not pass the QC validation. Additionally, this protocol shall be used if pre-made plates have been stored for a prolonged time and is aimed to be used for a new round of selective enrichment for ESBL and AmpC producing *E. coli*.

It is also important to emphasise that the these two strains are only meant as control strains for validation of the selective MacConkey agar plates and not as ESBL or AmpC control strains, as they have solely been chosen for their phenotypic characteristics in relation to the selective agar plates.

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Procedure

1. The two test strains (negative and positive control for plate validation procedures) supplied by the EURL AMR should be stored at -80 degree Celsius in glycerol upon arrival from the EURL AMR.

2. It is the intention that this protocol shall be used each time the protocols for selective enrichment of ESBL and AmpC producing *E. coli* in either fresh meat or caecal material from production animals are carried out or if different batches of MacConkey agar plates supplemented with 1 mg/L cefotaxime are used.

3. The two control strains are inoculated to single colonies from the -80 ºC freezing stock onto fresh blood agar plates and incubated at 37 ºC for 18-24 hours.

4. After incubation, the two test strains are resuspended in 0.9% saline separately and the cell density adjusted to McFarland 0.5.

5. Dilute each test strain a 1000-fold in fresh Buffered Peptone Water (BPW) and incubate 18-22 hours at 37 ºC.

6. After this incubation, a 10 µl loop is used to streak each of the control strain cultures on separate plates of MacConkey agar supplemented with 1 mg/L cefotaxime. This inoculation should be done in a single streak (1; Figure 1). From this streak, two additional streaks (2 and 3; Figure 1) are

Theory/comments

The activity of cefotaxime might decrease upon prolonged storage. Therefore, it is recommended that this protocol is used each time any of the two protocols for selective enrichment of ESBL and AmpC producing *E. coli* is performed.

Other nutrient agar plates can be used. However, blood agar is often helpful to identify bacterial contaminants based on colony morphology.

E.g. 9 µl McFarland-adjusted sample in 9 ml BPW or 10 µl in 10 ml BPW.

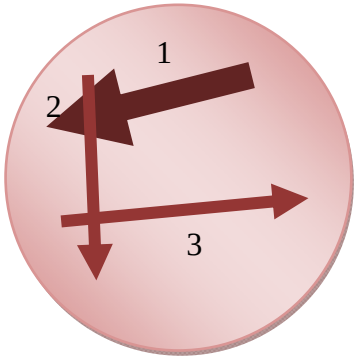

Figure 1

performed with 1 µl loops to obtain single colonies.

7. The MacConkey agar plates are incubated 18-22 hours at **44 ºC**.

8. After incubation of the plates, growth is assessed.

It is important to change the temperature in this step to simulate the incubation temperature used in the two protocols.

Full inhibition of growth should be obtained for the negative control. For the positive control it is expected to observe growth, and single colonies should be obtained in either streak 2 or streak 3 according to Figure 1.

If these results are not observed, the cefotaxime in the plates is probably not in the right concentration and therefore a revision of the procedures and troubleshooting is necessary.

Note, if only weak growth of the positive control is observed, it could be due to the type of MacConkey agar chosen and in this case a different variant of MacConkey agar should be considered.

If the negative control is able to grow on the plates, it could be considered to adjust the method by increasing the cefotaxime concentration, while - in case the positive control does not grow - it could be considered to decrease the concentration.

Any adjustments introduced as regards the media preparation at the national reference laboratories must be documented and validated.

APPENDIX 1

Composition and preparation of culture media and reagents

The Buffered peptone Water and MacConkey Agar media and reagents are available from several companies. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also, the media should be prepared according to the manufacturer's description if it differs from the description given here.

Buffered peptone water

Formula	g/litre
Enzymatic digest of casein	10.0
Sodium chloride	5.0
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	9.0
Potassium dihydrogen phosphate (KH_2PO_4)	1.5
pH 7.0 +/- 0.2 @ 25 °C	

Dissolve the components in water by heating if necessary. Adjust the pH, so that after sterilization it is 7.0+/- 0.2 at 25 °C. Dispense the medium into flasks of suitable capacity to obtain the portions necessary for the test. Sterilize for 15 min in the autoclave set at 121 °C.

MacConkey agar

Formula	g/litre
Pancreatic Digest of Gelatin	17.0
Peptones (meat and casein)	3.0
Lactose	10.0
Bile salts No. 3	1.5
Sodium chloride	5.0
Neutral red	0.03
Crystal violet	0.001
Agar	13.5
pH 7.1 +/- 0.2 @ 25 °C	

Suspend 50 g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

Selective Supplements

Formula	mg/mL
1. cefotaxime sodium salt stock solution prepared in bi-distilled water	1mg/mL

It is important to take the activity of the drug into account to ensure that 1 mg/mL active compound is used. Aliquots of aqueous cefotaxime stock solution (concentration 1mg/mL) can be stored at - 20°C.