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 caecal samples

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Version 3

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HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
3	Title	'caecal samples' instead of 'animals'	03 Nov 2017	Rene S. Hendriksen, Valeria Bortolaia, Susanne Karlsmose Pedersen
	Background	Frequency of performing validation specified		
	Item 2	Text deleted (moved to 'Background')		
	Item 6	Specified the use of 1-µL or 10-µL loop		
	Throughout the document	Editorial changes. Added acceptance interval for the incubation temperatures		
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
1	New document	-	21 Nov 2014	Authors

Background

For the harmonized monitoring of ESBL- and AmpC-producing *E. coli*, it is important 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.

Frequency of performing the test of validity of MacConkey agar plates supplemented with 1 mg/L cefotaxime

As the activity of cefotaxime might decrease upon prolonged storage, this protocol must be applied for validation of the agar plates prior to performing the analysis related to selective enrichment for ESBL- and AmpC-producing *E. coli* from meat and caecal samples.

Selective MacConkey agar plates supplemented with 1 mg/L cefotaxime must be tested with the negative and the positive control described in this protocol to determine if the performance obtained with the media is as expected (i.e., no-growth of the negative control and growth of the positive control on the media). If the performance is not as expected, the media batch should not be used for the purpose of selective isolation of ESBL-/AmpC-producing *E. coli.*

Validation of non-commercial MacConkey agar plates supplemented with 1 mg/L cefotaxime: 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 apply the protocol given below to ensure that the selected MacConkey agar (supplemented with the correct amount of cefotaxime) allows the positive test strain to grow while it prevents growth of the negative control strain.

Previous to using a new batch of non-commercial MacConkey agar plates supplemented with 1 mg/L cefotaxime for selective isolation of ESBL-/AmpC-producing *E. coli*, the batch must be validated by performing tests using the negative and positive controls as described in this protocol. Media yielding expected results (i.e. no-growth of the negative control and growth of the positive control) has passed the validation.

The frequency of additional validation of the same batch of media with negative and positive controls must be defined within each laboratory performing the analysis. It is proposed that the testing is performed each week to avoid the rejection of too many test results if validation fails.

If the results of a validation turn out to be unexpected (i.e. growth of the negative control or no-growth of the positive control on the selective media), all results obtained with this batch since the latest passed validation must be assessed further or rejected.

Commercially available MacConkey agar plates supplemented with 1 mg/L cefotaxime: The use of the media must be according to the manufacturer's descriptions/guidelines and the media must be used within the shelf life indicated by the manufacturer.

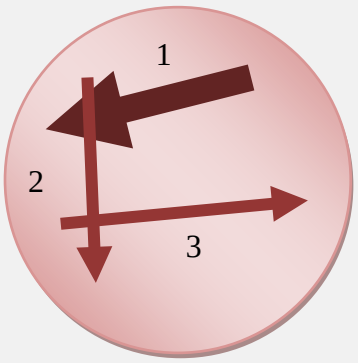
For commercially available MacConkey agar plates supplemented with 1 mg/L cefotaxime, the frequency of media validation with negative and positive controls must also be defined within each laboratory performing the analysis. It is proposed that the testing is performed each week to avoid the rejection of too many unacceptable test results.

If the results of a validation turn out to be unexpected (i.e. growth of the negative control or no-growth of the positive control on the selective media), all results obtained with this batch of media since the latest passed validation must be assessed further or rejected.

Negative and positive control strains

Based on expert advice, the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR) has identified and validated a set of test strains with cefotaxime MIC values close to the selective concentration of cefotaxime in the plates. The control set consists of two strains: a negative control and a positive control. Both of these strains were shipped to the National Reference Laboratories (NRLs) together with the External Quality Assurance System (EQAS) for *Salmonella* and *Campylobacter* in October 2014. These strains were labeled as a negative and a positive control, respectively, for 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.

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

Procedure		Theory/comments
1. The two test strains (negative and positive control for plate validation procedures) supplied by the EURL-AR should be stored in glycerol at -80°C upon arrival from the EURL-AR.		
2. -		*(Bullet-point deleted (moved to 'background'))*
3. Prepare a culture of the two control strains onto fresh blood agar plates from the -80°C freezing stock. Ensure to streak appropriately to obtain single colonies. Incubate the plates at 37°C ±1°C for 18-24 h.		Other nutrient agar plates can be used. However, blood agar is often helpful to identify bacterial contaminants based on presence of different colony morphologies.
4. After incubation, re-suspend each test strain separately in 0.9% saline and adjust the cell density to McFarland 0.5.		
5. Dilute each test strain 1000-fold in fresh buffered peptone water (BPW) and incubate at 37°C ±1°C for 18-22 h.		For example, 9 µL McFarland-adjusted sample in 9 ml BPW or 10 µL in 10 mL BPW.
6. After the incubation described in item 5, plate each culture on separate MacConkey agar plates (supplemented with 1 mg/L cefotaxime) as follows. Use a 10-µL loop of the overnight culture (item 5) to make a single streak ('1' in Figure 1). From this streak, perform two additional streaks ('2' and '3' in Figure 1) with a 1 µL or 10 µL loop to obtain single colonies (the same loop may be used for all three streaks).		 Figure 1

Procedure		Theory/comments
7. Incubate the streaked MacConkey agar plates at **44°C** ± 0.5°C for 18-22 h**.**		It is important to change the temperature in this step to simulate the incubation temperature used in the two protocols, 'Isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from fresh meat' and 'Isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from caecal samples'.
8. After incubation of the plates, assess the growth.		Full inhibition of growth should be obtained for the negative control. For the positive control, it is expected to observe growth: 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 the batch of plates should be assessed further or rejected. 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 grows on the plates or if the positive control does not grow on the plates, there are problems with concentration of cefotaxime. Troubleshooting should question any step from storage of cefotaxime powder and preparation of antibiotic stock solutions to influence of agar composition on cefotaxime potency. If media is purchased externally, troubleshooting should include contact to the manufacturer. **Any adjustments introduced with regards to media preparation at the NRLs must be documented and validated.**

APPENDIX 1

Composition and preparation of culture media and reagents

The buffered peptone water (BPW) 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 slightly 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/L
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 by autoclaving at 121°C for 15 minutes.

MacConkey agar

Formula	g/L
Pancreatic Digest of Gelatine	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 L distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Selective supplements

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

It is important to take the potency of the antimicrobial into account 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°C.
Example: If the manufacturer has stated a potency of 50%, 2 mg of antibiotic powder should be added to 1 mL of sterile dH_2O (or the solvent routinely used) to reach a final concentration of 1 mg/mL of active compound.