DTU Food
National Food Institute

LABORATORY PROTOCOL

Quantification of ESBL/AmpC-producing *Escherichia coli* in caecal content and fresh meat samples

December 2017
Version 1

Authored by the EURL-AR

HISTORY OF CHANGES				
Version	Sections changed	Description of change	Date	Approval
1	New document	-	18 December 2017	Valeria Bortolaia, René S. Hendriksen

Background

Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) and AmpC cephalosporinases are of major public health significance (1). In order to harmonize the antimicrobial resistance (AMR) monitoring systems in the European Union (EU), the European Commission (EC) adopted new legislation laying down detailed rules for the monitoring and reporting of AMR in zoonotic and commensal bacteria by Member States (MSs). The new legislation, 'Commission Implementing Decision on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria' (2013/652/EU) (2), includes the obligatory monitoring of ESBL- and AmpC-producing *E. coli* and the voluntary monitoring of carbapenemase-producing *E. coli* in meat and caecal samples, according to the most recent version of the protocol of the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AR).

Commission Implementing Decision 2013/652/EU also includes voluntary assessment of the proportion of ESBL-/AmpC-producing *E. coli* within the whole *E. coli* population of a sample according to the most recent version of the protocol of the EURL-AR (point 4.3 of the Annex). Within-sample quantification of ESBL-/AmpC-producing *E. coli* is particularly relevant for MSs that detected a high prevalence of samples positive for ESBL-/AmpC-producing *E. coli*.

The present protocol provides instructions for quantification of ESBL/AmpC- producing *E. coli* in caecum and fresh meat samples. It is the result of testing and validation on caecal content and meat samples from pigs, cattle and poultry performed by the EURL-AR at the Technical University of Denmark (Appendix 1). The protocol explains the procedure step-by-step and provides information on the theory behind each step.

Contents

IMPORTANT NOTES:

I. Samples must be kept refrigerated (5°C ± 3°C) at all times from collection to analysis. During analysis, ensure to keep the samples out of the refrigerator for the minimum amount of time possible and no longer than 15 minutes.

II. The time interval between collection of samples and start of analysis varies according to the matrix:

- Caecal content samples must be processed within 48 hours of collection.
- Meat samples must be processed within the expiration date as stated on the label

III. Each step must be performed in duplicate to increase accuracy of calculations of colony forming units (CFUs).

IV. This protocol is mainly designed to be applied in case of loads of ESBL/AmpC-producing *E. coli* in the range 10^3-10^8 CFU/g, thus is useful in those situation with high prevalence of samples positive for ESBL-/AmpC-producing *E. coli*. Given the current sampling procedures, high prevalence of samples positive for ESBL-/AmpC-producing *E. coli* is indicative of relatively high loads of ESBL/AmpC-producing *E. coli* (3).

V. This protocol is not intended for counts of carbapenemase-producing *E. coli* (CPE). At present, CPE occur at low prevalence in food animals and meat in EU Member States, thus highly sensitive methods such as selective enrichment are currently recommended for detection of CPE. Selective enrichment methods alter the relative proportion of bacteria within samples and therefore are not suitable to downstream calculations of CFUs.

Procedure		**Theory/comments**
1. Counts of colony forming units (CFUs) of ESBL/AmpC and total *E. coli*		
1.1 For caecal content samples, mix 1 g ± 0.1 g of sample in 9 ml of cold 0.9% saline. Vortex for at least 20 s to ensure adequate suspension. For meat samples, mix 25 g ± 0.5 g of sample in 225 ml of cold 0.9% saline in a plastic bag that can be tightly closed (e.g. a Stomacher bag). Shake vigorously for 50 times.		*Please refer to Appendix 2 and 3 for media composition and flow diagram of the entire procedure, respectively.* It is critical to vortex/shake thoroughly to ensure homogeneous cell suspension and, in case of meat samples, to ensure that cells detach from the meat surface. This is the 10^{-1} dilution of the sample. All dilutions should be completed and plated within 15 minutes to prevent artefacts such as variation in CFUs number.

Procedure		Theory/comments
1.2 By using a pipette, transfer 100 µl from the suspension at point 1.1 into an Eppendorf tube containing 900 µl of cold 0.9% saline. Discard the pipette tip. Vortex well.		This is the 10^{-2} dilution of the initial sample. When making dilutions to obtain decreasing concentrations of bacteria, pipette tips should be changed at each dilution step to avoid carryover of bacteria.
1.3 By using a new pipette tip, transfer 100 µl from the suspension at point 1.2 into an Eppendorf tube containing 900 µl of 0.9% saline. Discard the pipette tip. Vortex well.		This is the 10^{-3} dilution of the initial sample.
1.4 Proceed with serial dilutions until the desired final dilution (e.g. 10^{-6}). Remember to change pipette tip at each passage.		Typically, dilutions should be performed up to 10^{-6} to ensure to obtain countable plates for total *E. coli*
1.5 Once all the desired dilutions are ready, proceed with plating as follows. Plate 100 µl of each relevant dilution***** on MacConkey agar supplemented with 1 µg/ml cefotaxime or on a commercial agar suitable for growth of ESBL/AmpC-producing *E. coli*. *****to obtain countable plates (see theory at point 1.7), it is recommended to plate at least the dilutions: • from 10^{-1} to 10^{-4} for expected initial levels of 10^5 - 10^8 CFU/g. • from undiluted to 10^{-3} for expected initial levels of 10^3 - 10^5 CFU/g. • from 10^0 to 10^{-1} for expected initial levels of 10^1 and 10^2 CFU/g. In these cases of low prevalence of presumptive ESBL/AmpC-producing *E. coli*, false negatives may occur.		Plating can be performed using a Drigalski spatula, glass beads or similar appropriate methods routinely applied at the laboratory. A Quality Control (QC) procedure to validate cefotaxime-containing MacConkey agar plates produced in house should be performed according to EURL-AR recommendations (see www.eurl-ar.eu/protocols.aspx). If using commercially available selective plates, manufacturer's instructions should be followed.

Procedure		Theory/comments
Plate 100 µl of each relevant dilution** on MacConkey agar without antibiotic or another agar allowing isolation and identification of *E. coli*. **to obtain countable plates (see theory at point 1.7), it is recommended to plate at least the dilutions: • from 10^{-2} to 10^{-4} for expected initial levels of 10^5 and 10^8 CFU/g.		Plates without cefotaxime are used to calculate the total number of *E. coli*. All plates should be placed in the incubator within 15 minutes after inoculation to prevent artefacts such as variation in CFUs number. Initial levels of ESBL/AmpC-producing *E. coli* and total *E. coli* vary greatly with sample type (caecal content vs. meat) and local epidemiological situation. Drop plate technique might be an alternative for bacterial enumeration but has not been validated in this protocol. Laboratories who wish to perform CFU enumeration by the drop plate method should ensure to obtain counts similar to those obtained by the spread method.
1.6 Plates are incubated upside down at 44°C ± 0.5°C for 18-22 hours.		It is important that plates are incubated upside down to avoid artefacts by moisture on the agar surface (e.g. confluent growth which will hamper the possibility to count CFUs).
1.7 Colonies with typical *E. coli* appearance (red/purple on the MacConkey agar plates; Figure 1) are counted. Plates with > 300 and < 30 colonies should not be counted ("too many to count" and "too few to count", respectively). For final counts, an arithmetic average of the replicates of the countable dilution is used.		To ensure an accurate estimate of the number of *E. coli* cells (both ESBL/AmpC and total *E. coli*) in the original sample, plates with > 300 colonies and < 30 colonies should not be used for CFU counts.
2. Confirmation of *E. coli* species		
2.1 Individual colonies from the countable plates (both cefotaxime-supplemented and cefotaxime-free plates) should be used for verification of *E. coli* species. Depending on the method used for species identification, a subculture step might be necessary. In such		Different laboratories may have different methods (biochemical tests, mass spectrometry, chromogenic agar, genetic-based methods, etc.) for performing species identification of *E. coli*. The number of colonies selected depends on the laboratory's success rate of identifying *E. coli* on MacConkey agar

Procedure		Theory/comments
case, sub-culture individual colonies onto appropriate plates (i.e. MacConkey agar supplemented with 1 mg/L cefotaxime or another ESBL/AmpC selective medium for colonies originating from MacConkey+cefotaxime plates, and MacConkey agar or another medium supporting *E. coli* growth but without antimicrobial selective pressure for colonies originating from MacConkey agar plates. It is important to streak aiming to obtain single colonies to ensure pure culture. Incubate at 37°C ± 1°C for 18-22 h.		plates. It is recommended to test at least one presumptive *E. coli* colony for each morphology observed at item 1.7.
3. Phenotypic confirmation of ESBL/AmpC production in *E. coli*		
3.1 Confirmed *E. coli* colonies presumptively producing ESBL/AmpC should be subcultured on MacConkey agar supplemented with 1 µg/ml cefotaxime or another ESBL/AmpC selective medium. It is important to streak aiming to obtain single colonies to ensure pure culture. Incubate at 37°C ± 1°C for 18-22 h. NOTE: If the laboratory performed step 2.1, the subcultures obtained at that step on MacConkey agar with 1 µg/ml cefotaxime (or another ESBL/AmpC selective medium) and yielding confirmed *E. coli* can be used directly for step 3.2.		It is recommended to subculture at least one confirmed *E. coli* presumptively producing ESBL/AmpC for each morphology observed at item 1.7.
3.2 Confirmed *E. coli* presumptively producing ESBL/AmpC and in pure culture should be tested for antimicrobial susceptibility using		It is recommended to test at least one confirmed *E. coli* presumptively producing ESBL/AmpC for each morphology observed at item 1.7.

Procedure		Theory/comments
the panel of beta-lactam antimicrobials (Table 4 of Commission Implementing Decision 2013/652/EU).		
4. Quantification of ESBL/AmpC-producing *E. coli* in original samples		
4.1 The colony counts can be used to report: • CFU/g of ESBL/AmpC-producing *E. coli* in caecal content/meat samples • CFU/g of total *E. coli* in caecal content/meat samples • Proportion of ESBL- and AmpC-producing *E. coli* out of total *E. coli* in caecalcontent/meat samples.		Calculation of CFU/g of sample can be done as follows: • No. of CFU x 10 x dilution factor= CFU/ml • CFU/ml x 9 =cfu/g of caecal content or meat **Example** Caecal content from cattle: The two MacConkey+CTX plates on which 100 µl of 10^{-3} dilution were plated yield 60 and 96 colonies. The average is (60+96)/2=78, meaning there were 78 CFU in 100 µl which equals 780 CFU in 1 ml that can also be written as $7.8x10^{2}$ cfu/ml. This was the -3 dilution of the original culture, thus in the original culture there were $7.8x10^{5}$ cfu/ml. The culture had 1 g of caecal content in 9 ml of saline thus there were $7.8x10^{5}$ x9 cfu/g of caecal content, which equals to a load of presumptive ESBL/AmpC-producing *E. coli* of $7x10^{6}$ cfu/g of caecal content. Calculation of proportion of ESBL/AmpC-producing *E. coli* out of total *E. coli:* *Ratio between CFU calculated on CTX plates and CFU calculated on non-selective plates*

References

(1) Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. EFSA Journal 2011;9(8):2322 [95 pp.]. doi:10.2903/j.efsa.2011.2322.

(2) Commission Implementing Decision of 12 November 2013 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria (2013/652/EU).
OJ L 303, 14.11.2013, p. 26
http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1417790423875&uri=CELEX:32013D0652

(3) Vieira AR, Wu S, Jensen LB, Dalsgaard A, Houe H, Wegener HC, Lo Fo Wong DMA, Emborg H-D. Using data on resistance prevalence per sample in the surveillance of antimicrobial resistance, Journal of Antimicrobial Chemotherapy 2008;62(3):535-538
https://doi.org/10.1093/jac/dkn210

Figures

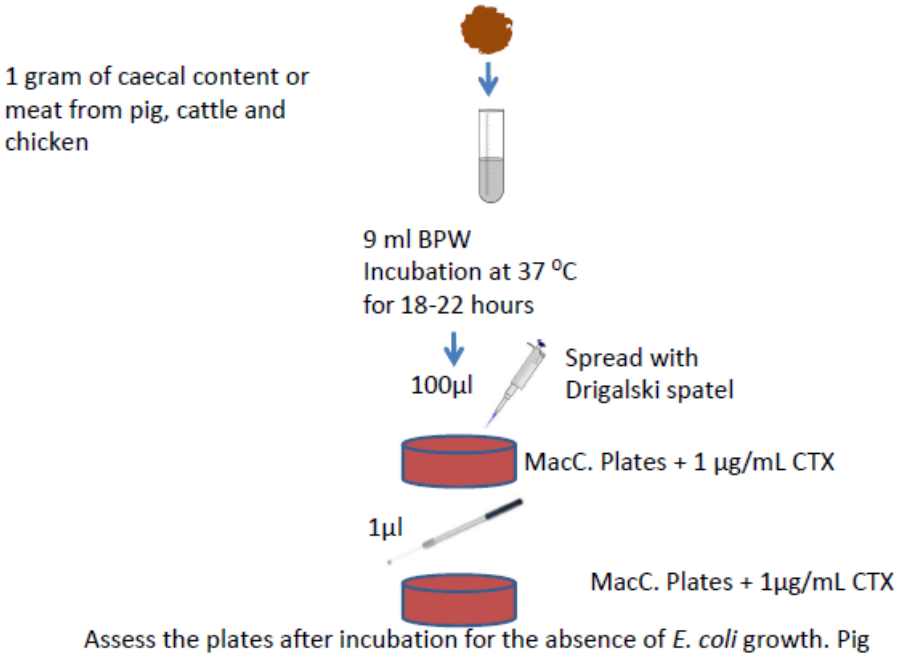
Figure 1: Typical appearance of *E. coli* on MacConkey agar supplemented with 1 mg/L cefotaxime.

APPENDIX 1

Validation protocol - Procedure

Step 1

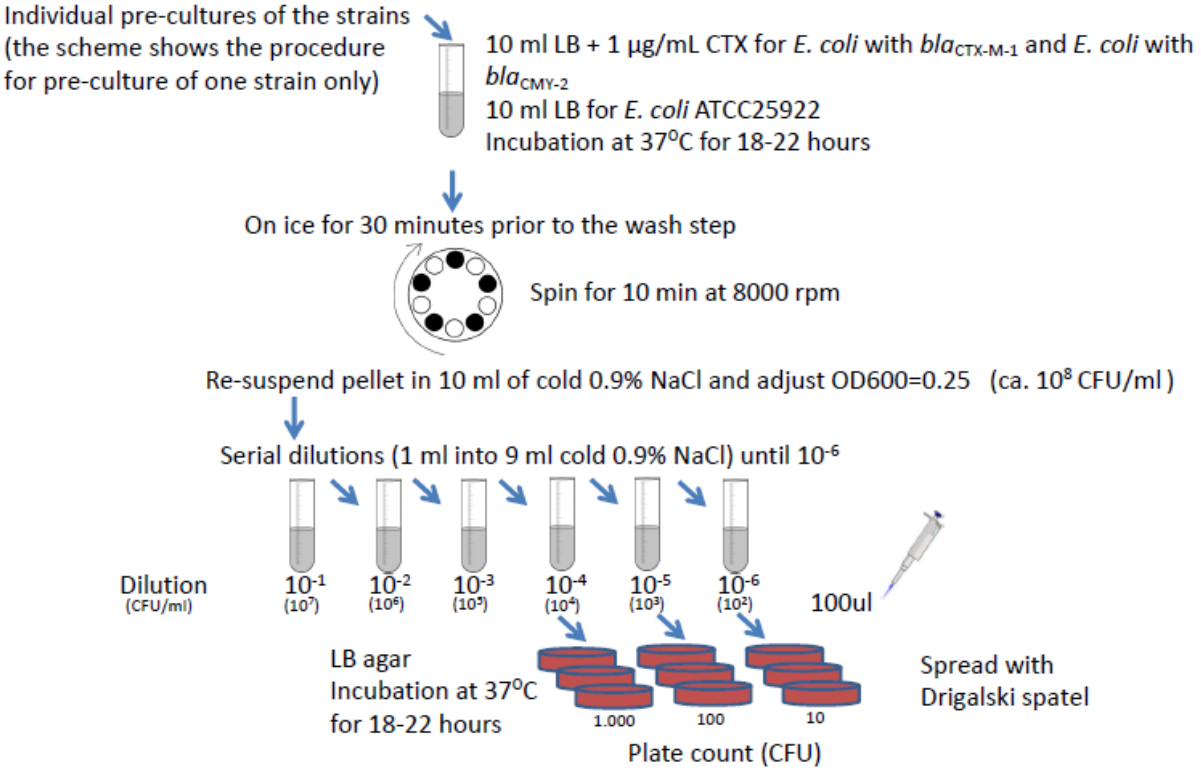
Pre-testing the caecal and meat samples



Assess the plates after incubation for the absence of *E. coli* growth. Pig caecal content samples represent an exception due to issues in survival of strains used for spiking. In this case, samples that were positive at the pre-test were used for validation.

Step 2

Preparing the cultures for spiking the samples



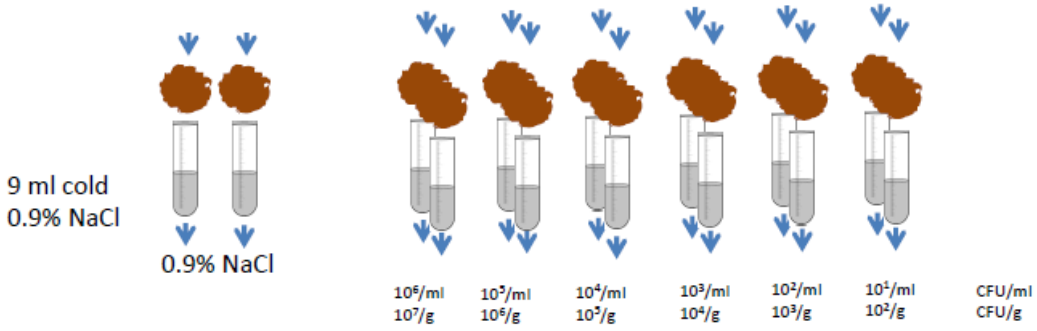
Step 3

Spiking the caecal and meat samples

A) 1 g of caecal content or meat in 9 ml 0.9% cold saline - each step is performed in duplicate

B) 1 ml of sterile 0.9% saline is added to one set of tubes

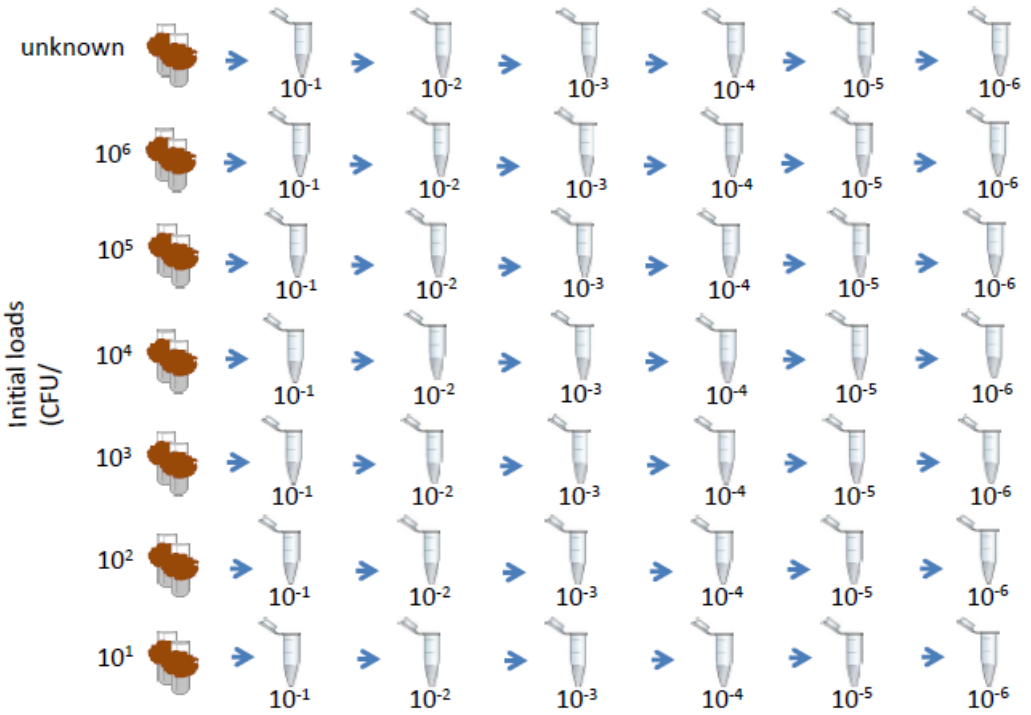
C) 1 ml of the different spiking dilutions is added to different sets of tubes to simulate different initial bacterial loads



Step 4

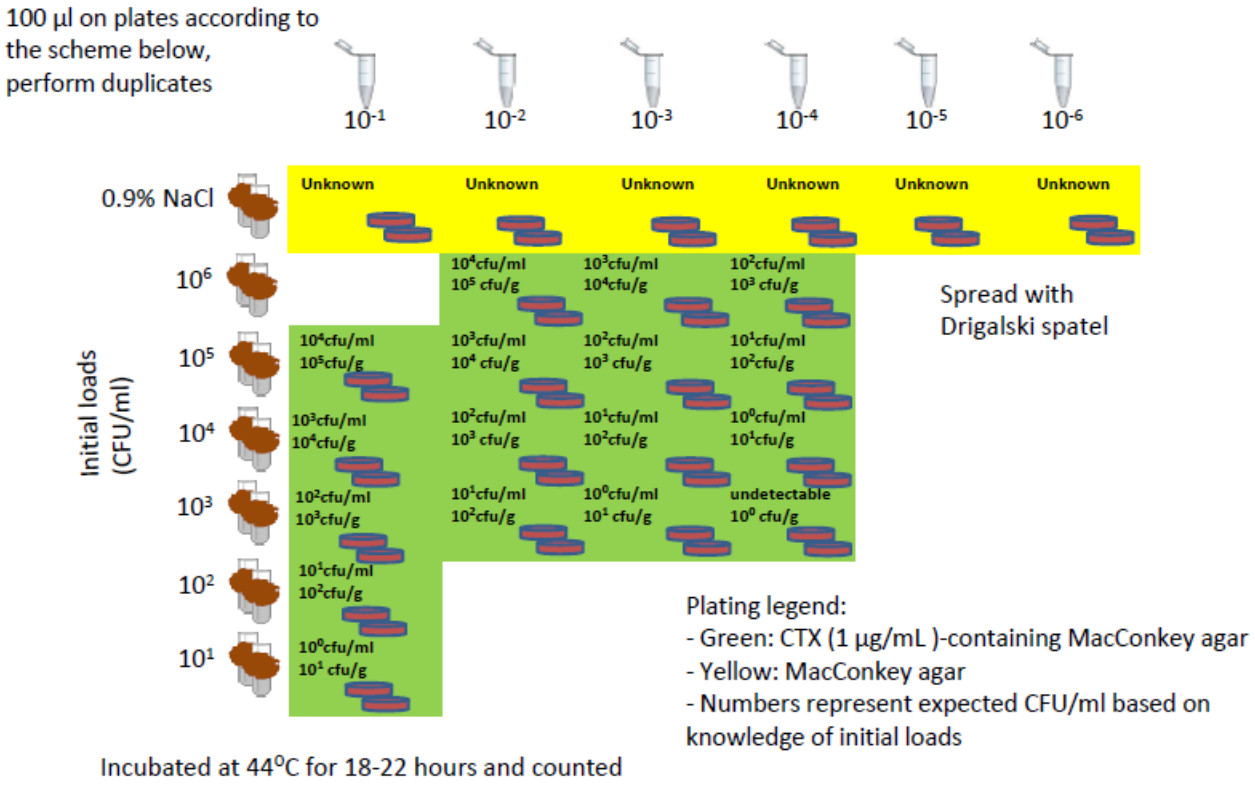
Serial dilutions of the samples (original and spiked)

Serial passages of 100 µl of solution in 900 µl of cold 0.9% saline



Step 5

Plating of relevant dilutions



Validation protocol – Results

Poultry samples

CFU counts, Validation experiment

E. coli with $bla_{CTX-M-1}$

Matrix									
Chicken meat					Chicken caecal content				
Initial load CTX-M-1-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)	Initial load CTX-M-1-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$1.23x10^{7}$	$4.9x10^{5}$	-3	0	none	$1.05x10^{7}$	$5.8x10^{5}$	-3	$1.58x10^{7}$	-4
$1.23x10^{6}$	not available	-1			$1.05x10^{6}$	$5.9x10^{4}$	-2		
$1.23x10^{5}$	not available	undiluted			$1.05x10^{5}$	not available	-1		
$1.23x10^{4}$	undetectable	none			$1.05x10^{4}$	not available	undiluted		
$1.23x10^{3}$	undetectable	none			$1.05x10^{3}$	undetectable	none		
$1.23x10^{2}$	undetectable	none			$1.05x10^{2}$	undetectable	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

E. coli with bla_{CMY-2}

Matrix									
Chicken meat					Chicken caecal content				
Initial load CMY-2-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)	Initial load CMY-2-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$7.63x10^{6}$	$1.74x10^{5}$	-2	0	none	$7.36x10^{6}$	$4.45x10^{5}$	-3	$1.43x10^{7}$	-4
$7.63x10^{5}$	not available	-1			$7.36x10^{5}$	$4.2x10^{4}$	-2		
$7.63x10^{4}$	not available	undiluted			$7.36x10^{4}$	not available	undiluted		
$7.63x10^{3}$	undetectable	none			$7.36x10^{3}$	undetectable	none		
$7.63x10^{2}$	undetectable	none			$7.36x10^{2}$	undetectable	none		
$7.63x10^{1}$	undetectable	none			$7.36x10^{1}$	undetectable	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

E. coli ATCC25922

Matrix									
Chicken meat					Chicken caecal content				
Initial load *E. coli* ATCC25922 (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)	Initial load *E. coli* ATCC25922 (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$1.43x10^{7}$	0	none	0	none	$1.05x10^{7}$	0	none	$1.71x10^{7}$	-4
$1.43x10^{6}$	0	none			$1.05x10^{6}$	0	none		
$1.43x10^{5}$	0	none			$1.05x10^{5}$	0	none		
$1.43x10^{4}$	0	none			$1.05x10^{4}$	0	none		
$1.43x10^{3}$	0	none			$1.05x10^{3}$	0	none		
$1.43x10^{2}$	0	none			$1.05x10^{2}$	0	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

Validation protocol – Results

Pig samples 1/2

CFU counts, Validation experiment

E. coli with $bla_{CTX-M-1}$

Matrix									
Pork					Pig caecal content				
Initial load CTX-M-1-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)	Initial load CTX-M-1-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$9.23x10^{6}$	$1.93x10^{5}$	-2	0	none	$1.94x10^{7}$	$8.6x10^{5}$	-3	$1.06x10^{6}$	-3
$9.23x10^{5}$	not available	-1			$1.94x10^{6}$	$1.22x10^{5}$	-2		
$9.23x10^{4}$	not available	undiluted			$1.94x10^{5}$	not available	-1		
$9.23x10^{3}$	undetectable	none			$1.94x10^{4}$	not available	undiluted		
$9.23x10^{2}$	undetectable	none			$1.94x10^{3}$	undetectable	none		
$9.23x10^{1}$	undetectable	none			$1.94x10^{2}$	undetectable	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

E. coli with bla_{CMY-2}

Matrix				
Pork				
Initial load CMY-2-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$5x10^{6}$	$2.53x10^{5}$	-2	0	none
$5x10^{5}$	$3.05x10^{4}$	-2;-1		
$5x10^{4}$	not available	-1		
$5x10^{3}$	not available	undiluted		
$5x10^{2}$	undetectable	none		
$5x10^{1}$	undetectable	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

E. coli ATCC25922

Matrix				
Pork				
Initial load *E. coli* ATCC25922 (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$6.76x10^{6}$	0	none	0	none
$6.76x10^{5}$	0	none		
$6.76x10^{4}$	0	none		
$6.76x10^{3}$	0	none		
$6.76x10^{2}$	0	none		
$6.76x10^{1}$	0	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

Validation protocol – Results

Pig samples 2/2

Matrix				
Pig caecal content				
Initial load ESBL/AmpC *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)[2]	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
unknown	$8x10^{3}$	-1	$8.45x10^{5}$	-2

[1]the sample contained presumptive ESBL/AmpC-producing E. coli. No spiking was performed in this case.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

Matrix				
Pig caecal content				
Initial load ESBL/AmpC *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)[2]	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
unknown	$4x10^{1*}$	undiluted	$5.6x10^{5}$	-2
unknown	$2x10^{2*}$	-1		

[1]the sample contained presumptive ESBL/AmpC-producing E. coli. No spiking was performed in this case.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

*the low number of colonies recovered is within the non-countable range (<30). Thus, the sample is positive for ESBL/AmpC-producing E. coli at level ≤ 10^{2} CFU/g

Validation protocol – Results

Bovine samples

CFU counts, Validation experiment

E. coli with *bla* $_{CTX-M-1}$

Matrix									
Beef					Cattle caecal content				
Initial load CTX-M-1-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)	Initial load CTX-M-1-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$8.5x10^6$	$4.6x10^6$	-4	0	none	$1.53x10^7$	$7.8x10^5$	-3	$4.15x10^4$	-2
$8.5x10^5$	$3x10^5$	-3			$1.53x10^6$	$8.8x10^4$	-2		
$8.5x10^4$	$3.8x10^4$	-2			$1.53x10^5$	not available	-1		
$8.5x10^3$	not available	-1			$1.53x10^4$	not available	undiluted		
$8.5x10^2$	not available	undiluted			$1.53x10^3$	undetectable	none		
$8.5x10^1$	undetectable	none			$1.53x10^2$	undetectable	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

E. coli with *bla* $_{CMY-2}$

Matrix									
Beef					Cattle caecal content				
Initial load CMY-2-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)	Initial load CMY-2-producing *E. coli* (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$6.53x10^6$	$6.35x10^6$	-4	0	none	$1.41x10^7$	$7x10^5$	-3	$3.75x10^4$	-2
$6.53x10^5$	$5.45x10^5$	-3			$1.41x10^6$	$6x10^4$	-2		
$6.53x10^4$	$2.85x10^4$	-2			$1.41x10^5$	not available	-1		
$6.53x10^3$	not available	-1			$1.41x10^4$	not available	undiluted		
$6.53x10^2$	not available	undiluted			$1.41x10^3$	undetectable	none		
$6.53x10^1$	undetectable	none			$1.41x10^2$	undetectable	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

E. coli ATCC25922

Matrix									
Beef					Cattle caecal content				
Initial load *E. coli* ATCC25922 (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)	Initial load *E. coli* ATCC25922 (CFU/ml)[1]	Recovered load (MacConkey+CTX) (CFU/ml)	Dilution(s) yielding countable plates (30-300 CFU)	Initial load total *E. coli* (CFU/ml)[2]	Dilution(s) yielding countable plates for total *E. coli* (30-300 CFU)
$1.11x10^7$	0	none	0	none	$1.52x10^7$	0	none	$4.45x10^4$	-2
$1.11x10^6$	0	none			$1.52x10^6$	0	none		
$1.11x10^5$	0	none			$1.52x10^5$	0	none		
$1.11x10^4$	0	none			$1.52x10^4$	0	none		
$1.11x10^3$	0	none			$1.52x10^3$	0	none		
$1.11x10^2$	0	none			$1.52x10^2$	0	none		

[1]as calculated from the spiking solution. Given the sampling design the CFU/g of meat and caecal content is 10 x higher than the indicated CFU/ml. The pre-test showed that all samples were negative for ESBL/AmpC-producing E. coli.

[2]as calculated from non-spiked samples. Given the sampling design the CFU/g of meat and caecal content is 10x higher than the indicated CFU/ml.

APPENDIX 2

Composition and preparation of culture media and reagents

The 0.9% saline, 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. Of note, the media should be prepared according to the manufacturer's instructions, if they differ from the description given here.

0.9% saline

Formula	g/L
Sodium chloride (NaCl)	9

Dissolve in distilled water to a total volume of 1 L. Sterilize by autoclaving at 121°C for 15 minutes.

MacConkey agar (Example)

Formula	g/L
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 L of distilled water (Optional: Add 6.5 g agar to increase the hardness of the agar plates). 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 drug 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.

APPENDIX 3

FLOW DIAGRAM

for quantification of ESBL- and AmpC- producing *Escherichia coli* from caecal content and fresh meat samples

Counts of colony-forming units (CFUs) of ESBL/AmpC and total *E. coli* [item 1.1-1.7]

1 g of caecal content sample in 9 ml of cold 0.9% saline. Vortex thoroughly [item 1.1]

25 g of meat sample in 225 ml of cold 0.9% saline. Shake vigorously [item 1.1]

Go to next step within maximum 15 minutes

↓

Serial dilutions [item 1.2-1.4]

➔ 100 µl of either solution from item 1.1 in 900 µl of cold 0.9% saline. Vortex thoroughly [item 1.2]

➔ 100 µl of solution from item 1.2 in 900 µl of cold 0.9% saline. Vortex thoroughly [item 1.3]

➔ Proceed with four additional series of 1:10 dilutions to reach 10^{-6} dilution of the initial sample. Such dilution should provide countable number of colonies in most situations but local adjustments might be necessary [item 1.4]

Go to next step within maximum 15 minutes

↓

Plating [item 1.5-1.6]

➔ 100 µl of each relevant dilution (see protocol for details) onto a separate MacConkey agar plate with 1 mg/L cefotaxime [item 1.5]

➔ 100 µl of each relevant dilution (see protocol for details) onto a separate MacConkey agar plate without antibiotic [item 1.5]

➔ Incubate at 44 °C, 18-22 h [item 1.6]

Incubate plates within maximum 15 minutes from inoculation

↓

Colony counts [item 1.7] and *E. coli* species identification [item 2.1]

➔ Count *E. coli* colonies on countable plates
➔ Verify species ID by use of appropriate method

↓

Phenotypic confirmation of ESBL/AmpC production in *E. coli* [item 3.1-3.2]

➔ Obtain pure cultures of confirmed *E. coli* colonies growing on the countable MacConkey agar plate with 1 mg/L cefotaxime. One colony per morphology should be tested [item 3.1]

➔ Confirmed E. coli in pure culture and presumptively producing ESBL/AmpC from item 3.1 (or item 2.1 if the procedure for *E. coli* species identification included subculture) should be tested for antimicrobial susceptibility using the panel of beta-lactam antimicrobials [item 3.2]

Quantification of ESBL/AmpC-producing *E. coli* and total *E. coli* [item 4.1]

➔ Estimate number of colony forming units (CFUs) of ESBL/AmpC-producing *E. coli* per gram of starting material

➔ Estimate number of colony forming units (CFUs) of total *E. coli* per gram of starting material

➔ Estimate proportion of ESBL/AmpC-producing *E. coli* within total *E. coli* population in the specific sample