DTU Food
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VALIDATION REPORT

Validation of prolonged storage of samples before analysis for the monitoring of ESBL-, AmpC- and carbapenemase-producing *E. coli* from caecal samples

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Version 1

Anne Mette Seyfarth and Valeria Bortolaia, EURL-AR

Background

In November 2017, the laboratory protocol "Isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from caecal samples" was revised concerning the acceptable storage period of samples prior to analysis.

The new version of the protocol made it possible for laboratories to store caecal material arriving late Thursdays and Fridays over the weekend and start the analysis for ESBL-, AmpC- and carbapenemase-producing *E. coli* on Mondays, thus avoiding any work in the weekend.

The aim was to ensure that sampling for the harmonized EU surveillance for AMR could be equally performed over all five-business days of the week, including Thursdays and Fridays, which in many countries were not included as sampling days, because of the need for three consecutive working days at the laboratory for the isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli.*

The prolonged acceptable storage period up to 96 hours for the Thursday and Friday samples was introduced immediately, prior to a validation study, in order to ensure that changes in the sampling program for each country could be implemented already from January 2018.

When originally establishing the protocol, a storage period of 48 hours was adopted as a natural consequence of the needed transportation time from slaughterhouse to laboratory. The present document provides an assessment of the impact of additional 48 hours of storage of caecal samples before onset of analysis.

Data for the validation

The present validation includes results from studies performed at the EURL-AR laboratory (Part I), as well as a study of the Danish AMR surveillance data submitted to EFSA for the years 2016 - 2018 (Part II).

Part I

Prior to each launch of the annual EQAS on detection of ESBL-, AmpC- and carbapenemase-producing *E. coli* from caecal samples, a study has been performed to assess recovery of the selected *E. coli* test strains over time.

These preparational studies comprise three studies for pig caecal samples and three studies for poultry caecal samples. Each study includes 1) pre-testing of the caecal samples to identify negative samples, 2) spiking of negative samples with the selected *E. coli* test strains, 3) recovery of the *E. coli* test strains after storage of the EQAS samples for a pre-defined number of days, and finally, 4) verifying the recovered test strains by MIC-testing.

In details, the protocol for detection of ESBL-, AmpC- and carbapenemase-producing *E. coli* from caecal samples is applied to investigate recovery of test strains at Day 0, 2, 7 and 14 after spiking. Spiking is performed at an expected level of 10^3-10^4 CFU per gram of sample. Preferably, the selected *E. coli* test strains originate from the same animal species as the caecal material chosen for the specific EQAS.

The selected *E. coli* test strains for the pig and poultry studies primarily carried beta-lactam resistance genes of the type CMY-2, CTX-M, TEM and SHV and for all studies recovery of the test strains was successfully achieved all days of testing.

Recently, a study on cattle caecal samples was performed with the specific purpose to validate the prolonged storage period of samples for up to 96 hours (i.e. 4 days). For this study, caecal material from 10 different animals was included to account for background microbiota variations. Furthermore, *E. coli* strains resistant to third-generation cephalosporins and/or carbapenems via diverse genes were included (Table 1) The study was performed following the methods for preparation of a matrix EQAS, except for the fact that an additional day of analysis, Day 5 after spiking, was included in addition to day 0, 2, 7 and 14. Day 0 is a positive control for the spiking of samples, and Day 5 corresponds to 120 hours of storage before analysis, which is the closest to 96 h storage that could be achieved due to the work routine in the laboratory. This was based on the assumption that strains recovered at 120 h after spiking would also be isolated at 96 h after spiking.

The CFU used for spiking turned up to be unexpectedly low (Table 1). Nevertheless, the experiments were continued as in real-life scenarios ESBL-, AmpC- and carbapenemase-producing *E. coli* occur at unknown prevalence and likely constitute a limited proportion of the total *E. coli* population. In the vast majority of *E. coli* strain/sample combination tested, detection of the *E. coli* strains introduced for spiking was easily obtained after storage of the samples for up to 14 days (Table 1). Numerous colonies of the test strains grew on the selective agars, thus showing that recovery of the relevant strains was straightforward.

Two exceptions were observed. In one case using poultry caecal samples, an attempt to recover a test *E. coli* strain encoding bla_{KPC-2} (conferring carbapenemase phenotype) failed as the strain could only be

detected at Day 0 after spiking. Noteworthy, an alteration of the storage period from 48 h to 96 h would not have influenced the outcome. In two cases using cattle caecal samples, recovery of a test strain with mutations compatible with overexpression of chromosomal *ampC* (conferring AmpC phenotype) was unreliable (Table 1). The test strain was used for spiking of two samples and at Day 0, recovery failed in both samples. On the following days, recovery varied, but when successfull, it consisted of only one-two colonies on the selective agars. This could be explained by an unexpected low spiking concentration achieved for this particular strain (Table 1). To verify this, an additional experiment was performed using the test strain at a higher spiking concentration. Recovery was investigated at Day 5 and came out successfully with appearance of more colonies than observed in the first trial.

This example demonstrates that recovery of test strains is more influenced by the number of CFU present than by the time of storage before analysis.

Even if all of the above mentioned studies encompass a relatively limited number and variation of samples and test strains, the results are consistent and indicate that storage of samples for additional 48 hours, i.e. for a total of 96 h from sampling to onset of analysis, does not influence the prevalence of caecal samples positive for ESBL-, AmpC- and carbapenemase-producing *E. coli* within the framework of the EU AMR surveillance program.

Part II

In addition to the validation studies performed in the EURL-AR laboratory, an analysis of the Danish data submitted to EFSA for the EU surveillance program for the years 2016-2018 was performed.

The Danish data regarding occurrence of ESBL- and AmpC-producing *E. coli* in 2016 and 2018 (poultry surveillance) and in 2017 (pigs and cattle surveillance) were made available by the Danish authorities. These data provide a strong material for investigating the impact of a prolonged storage since the ESBL- and AmpC-producing *E. coli* detected were naturally occurring in real samples.

The data were used for comparing the prevalence of positive samples among Friday samples to the prevalence of positive samples among non-Friday samples. The samples received at the laboratory late Thursdays or Fridays (Friday samples), were sampled Wednesday/Thursday the same week, but processed in the laboratory on the following Mondays. The storage period of these samples was then 4-5 days before onset of the analysis, while the storage period of samples from other days of the week was 1-3 days.

Calculations were carried out both for each animal species per year and for the total dataset across the years. Data is not presented in details in this report, but it can be informed that 1) the data comprised at least 300 samples per year for each production animal sampled, 2) sampling was performed on all 5 working days and, in total, Friday samples represented 24 % of the samples (varying from 7 to 40 % depending on the animal species sampled), and 3) the observed prevalence of ESBL- and AmpC-producing *E. coli* in caecal samples from pigs, poultry and cattle was 25, 15 and 7 %, respectively.

For all the calculations, no significant differences (Fisher's exact test, p-values 0.43-0.70) in the detection of ESBL- and AmpC-producing *E. coli* in the Friday samples compared to non-Friday samples were observed.

Conclusion

In conclusion, the validation studies have demonstrated that, at least for the specific samples and *E. coli* test strains and spiking concentrations used, the detection of ESBL-, AmpC- and carbapenemase-producing *E. coli* in caecal samples of pigs, poultry and cattle could easily be achieved after 96 hours of storage prior to analysis.

These findings were supported by the investigation of the Danish dataset submitted to EFSA for the EU surveillance program for the years 2016-2018, which demonstrated that the prevalence of positive samples was not significantly affected by storage of the samples for 96 hours compared to 48 hours.

Table 1. Results for the validation study on recovery of *E. coli* from cattle caecal samples.

***E. coli* test strain**		**CFU for spiking**	**Sample**	**Recovery**				
Phenotype	**Genotype**			**Day 0**	**Day 2**	**Day 5**	**Day 7**	**Day 14**
AmpC	Upregulated chromosomal *ampC*	50 CFU/sample*	2	-	-	x	-	x
			7	-	x	x	-	-
ESBL	$bla_{CTX\text{-}M\text{-}1}$	500 CFU/sample	1	x	x	x	x	x
			6	x	x	x	x	x
ESBL	$bla_{CTX\text{-}M\text{-}2}$	300 CFU/sample	3	x	x	x	x	x
			8	x	x	x	x	x
CPE	$bla_{NDM\text{-}1}$	800 CFU/sample	4	x	x	x	x	x
			9	x	x	x	x	x
CPE	$bla_{OXA\text{-}162}$	200 CFU/sample	5	x	x	x	x	x
			10	x	x	x	x	x

*ESBL, Extended-Spectrum Beta-Lactamase; AmpC, AmpC-type beta-lactamase; CPE, carbapenemase-producing Enterobacteriaceae. *) A second test was performed with spiking of 100 CFU in Sample 2 and recovery was investigated at Day 5. Recovery was successful with appearance of more colonies than in the first trial.*

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