

NEWSLETTER

to the
**National Reference Laboratories
for Antimicrobial Resistance**

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Updated protocols for isolation of ESBL-, ampC- and carbapenemase-producing *E. coli*

By *Rene S. Hendriksen*

The EURL-AR has recently updated the EURL laboratory protocols for isolation of ESBL, ampC and carbapenemase-producing *E. coli* from fresh meat and caecal samples as well as the EURL laboratory protocol for validation of selective MacConkey agar plates supplemented with 1 mg/L cefotaxime.

During the EURL-AR workshops the last couple of years, there has been eager discussions on the sampling and storage of samples described in the protocols, indicating that the 48-hour rule (the time from sampling to initiated analysis) was too strict and induced additional work and costs. Based on their audits in Member States (MS), Directorate F reported that in some MSs, a lack of compliance with the Decision related to the sample storage time also caused a skewed sampling over the five business days. To accommodate some of these issues, the protocols were revised, prolonging the permitted sample storage time for samples taken Thursdays and Fridays to up to 96 hours.

The protocol has been validated for storage of samples in the laboratory for up to 24 hours. Validation of the 96-hour time interval between collection and analysis of caecal samples is planned to be performed by the EURL-AR in 2018. The update will take into consideration a possible loss of sensitivity due to possible overgrowth of completing microflora.

The EURL-AR/EFSA Reference Testing

By Valeria Bortolaia, Rene S. Hendriksen, Jette S. Kjeldgaard, Raquel Garcia Fierro, Pierre-Alexandre Boleil, Beatriz Guerra

The European Food Safety Authority (EFSA) and the European Reference Laboratory for Antimicrobial Resistance (EURL-AR) have performed a reference testing on a subset of isolates collected within the framework of Commission Implementing Decision 2013/652/EU since 2015.

Each year, *Escherichia coli* and *Salmonella* spp. isolates are chosen based on selection criteria agreed between the EFSA and the EURL-AR. Such criteria are defined mainly to validate results of emerging resistance and unusual phenotypes and to detect the corresponding mechanisms of resistance, and thus are regularly updated.

In 2015, 165 isolates from 24 European countries were selected with focus on:

- i) cephalosporin and/or carbapenem resistance also with the purpose to validate the rules used to infer presumptive ESBL/AmpC phenotypes;
- ii) colistin, azithromycin, and tigecycline resistance;
- iii) discrepant results between Panel 1 and Panel 2;
- iv) emerging resistant *Salmonella* serovars (*S. Infantis* and *S. Kentucky*).

In 2016, 187 isolates from 27 European countries were selected based on criteria similar to those followed in 2015 at points i), ii) and iii).

In 2017, 308 isolates from 30 European countries were selected with focus on:

- i) carbapenem-resistance;
- ii) colistin, azithromycin, and tigecycline resistance;
- iii) discrepant results between Panel 1 and Panel 2;
- iii) confirmation of inferred presumptive phenotypes for ESBL, AmpC, ESBL+AmpC, and carbapenemases (AmpC+ESBL-phenotype, AmpC phenotype with cefepime MIC=16 mg/L; ESBLs, AmpC)
- iv) country-specific issues;
- v) detection of emerging resistance mechanisms/clones: *S. Enteritidis* colistin-S vs. colistin-R with unknown colistin-R mechanisms.

The EURL-AR will receive all isolates after signing a Material Transfer Agreement with each National Reference Laboratory for Antimicrobial Resistance (NRL-AR). All isolates undergo i) antimicrobial susceptibility testing by minimum inhibitory concentration (MIC) determination according to European Commission Decision 2013/652/EU using the Trek Diagnostic Sensititre system; and ii) whole genome sequencing (WGS). Antimicrobial resistance genes will be detected using ResFinder (<https://cge.cbs.dtu.dk/services/ResFinder/>). Phylogenetic analyses are created using the pipeline CSI phylogeny available from CGE <https://cge.cbs.dtu.dk//services/all.php>.

Results will be analyzed by comparing phenotypes obtained by NRLs-AR and EURL-AR and by determining agreement between phenotypes and genotypes. In case of discrepancies, a thorough follow-up is initiated. Discrepant results might indicate presence of contamination in the culture, switch of strains, problems in defining MICs of specific antimicrobials such as sulfamethoxazole and azithromycin, and/or technical problems as reported by the EURL-AR for tigecycline and colistin. In selected circumstances and in agreement with the NRLs-AR concerned, non-confirmed results may eventually be either replaced with the reference results issued by the EURL-AR or excluded from the European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food (EUSR-AR report). In any case, the altered/suppressed results should be resubmitted to the EFSA as soon as possible.

In addition, at the EFSA network meeting on antimicrobial resistance (AMR) monitoring of 8-9 November 2017, it was agreed that selection criteria for reference testing will be circulated to the Member States (MSs) at the beginning of first semester of the year of reporting so that they can be used by the MSs to validate AMR data before reporting to EFSA. The importance of reporting representative and fully validated AMR data to EFSA should still be underlined.

NRLs-AR are therefore invited to use the reference testing results to perform an *a posteriori* (as well as in the future an *a priori*) self-evaluation and are welcome to contact the EURL-AR for further discussions.

Multiplex PCR for *mcr*-detection

By Ana Rita Rebelo and Jette S. Kjeldgaard

Since last year's communication on plasmid-mediated colistin resistance (Newsletter No. 10, December 2016) three more plasmid-borne *mcr* genes have been described: *mcr-3* (Yin et al., 2017), *mcr-4* (Carattoli et al., 2017) and *mcr-5* (Borowiak et al., 2017). Additionally, there are thirteen currently known variants of these genes present in *Enterobacteriaceae* (*mcr-1.2* to *mcr-1.9*, *mcr-1.11*, *mcr-3.2* to *mcr-3.4* and *mcr-3.7*) and there have been reports of co-occurrence of different *mcr* genes in the same isolates (Litrup et al., 2017, Hernández et al., 2017).

We developed a multiplex PCR (mPCR) that allows the detection of the five *mcr* genes and their variants in a rapid and easy way, which will hopefully become an important tool when screening for colistin resistance. We designed four sets of primers for amplifying *mcr-1-4* and used primers for *mcr-5* previously described by the original authors, obtaining an mPCR with a separation of amplicons of approximately 200 bp. The method was tested and validated in a sub-collection of strains recovered from the EFSA/EURL-AR confirmatory testing for 2016. The mPCR was able to successfully identify 100% of the *mcr* genes present in the strains according to WGS data, and also detected co-occurrence of *mcr-1/mcr-3* and *mcr-1/mcr-4*. During the data analysis we discovered two new *mcr-4* variants (*mcr-4.2* and *mcr-4.3*) and detected *mcr-1* in a phenotypically susceptible strain (MIC = 2 mg/L), indicating the need to review current classification standards.

We believe this method will positively impact epidemiological surveillance in areas where plasmid-borne colistin resistance is spreading and threatening the efficacy of antibiotic therapy. Furthermore, it can be a powerful complement to phenotypical antimicrobial resistance tests, due to the difficulty associated with the testing for colistin (http://www.eucast.org/ast_of_bacteria/warnings/) and the discovery of the existence of genes in clinically susceptible strains.

The publication is expected to be available in *Eurosurveillance* by early February 2018. Subsequent to this, a laboratory multiplex PCR protocol will be available on the EURL-AR website. For further information, please contact Jette S. Kjeldgaard (jetk@food.dtu.dk)

Further reading:

Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, et al. Novel plasmid-mediated colistin resistance gene *mcr-3* in *Escherichia coli*. *MBio*. 2017;8(3):e00543-17.

Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, et al. Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill*. 2017;22(31):pii=30589.

Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B. Identification of a novel transposon-associated phosphoethanolamine transferase gene, *mcr-5*, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. *J Antimicrob Chemother*. 2017; dkx327

Litrup E, Kiil K, Hammerum AM, Roer L, Nielsen EM, Torpdahl M. Plasmid-borne colistin resistance gene *mcr-3* in *Salmonella* isolates from human infections, Denmark, 2009–17. *Euro Surveill*. 2017;22(31):pii=30587.

Hernández M, Iglesias MR, Rodríguez-Lázaro D, Gallardo A, Quijada NM, Miguela-Villoldo P, et al. Co-occurrence of colistin-resistance genes *mcr-1* and *mcr-3* among multidrug-resistant *Escherichia coli* isolated from cattle, Spain, September 2015. *Euro Surveill*. 2017;22(31):pii=30586

EURL-AR Training Course 2017

By Jette S. Kjeldgaard

In September 2017, the EURL-AR at DTU Food hosted a training course in Denmark on the transition from genotype to phenotype in antimicrobial susceptibility testing (AST). The course was intended to cover the whole series of challenges that might occur, when a laboratory decides to strengthen the classical phenotypic AST with monitoring by whole genome sequencing (WGS).

The course covered a broad range of topics, including how to convince the management to invest in the technology, an overview of current technologies and technical and practical considerations. The course also included input from several member states (MS), at different levels of the implementation of WGS, break-out sessions for development of an implementation plan and hands-on computer exercises in genome analysis.

A total of 33 participants from 27 countries found their way to DTU Campus in Kgs. Lyngby for the three-day course, and the participants generally appreciated the relevant information and skills gained during the course and expressed that the training course was useful in introducing and providing background for implementation of sequencing and genome analysis at the MS level. Some of the outputs were a milestone plan for implementation of the technique and the requirements that should be considered by the NRLs as well as a basic understanding of AMR monitoring by screening for resistance genes.

The incitement for the EURL-AR to execute a course on this topic was to raise awareness of the many benefits of WGS in the monitoring of AMR. The collection of isolate sequences and resistance genotypes is a valuable tool for monitoring emerging resistances and for identifying the genotypes responsible for unusual phenotypes. Furthermore, generation of a database of veterinary isolates collected in the MS is fundamental in surveillance and trace-back of new resistance genes, when these are identified. An example of this is the identification of new colistin resistance mediating *mcr* genes and gene variants in veterinary isolates of *E. coli* and *Salmonella* in several European countries.

Presentations from the course are available for download on the EURL homepage:

<https://www.eurl-ar.eu/presentations/training-course-kgs-lyngby-whole-genome-sequencing-september-2017.aspx>

Did you notice:

- ⇒ [Fact sheet](#) on the new EU Action Plan on Antimicrobial Resistance
- ⇒ [E-learning course](#) on Whole Genome Sequencing (WGS) of Bacterial Genomes – tools and application
- ⇒ [Resistome Tracker](#), an Interactive Research and Data Visualization Tool for Antibiotic Resistance Genes launched by FDA
- ⇒ Drug-Resistant Infections – A Threat to Our Economic Future (A [World Bank Report](#))

Working Group on NGS

A Working Group (WG) on Next Generation Sequencing (NGS) coordinated by the EURL for VTEC has been established among the EURLs of *Listeria monocytogenes*, *Salmonella*, coagulase positive staphylococci, *Campylobacter*, Parasites, AMR and Food-borne viruses. The aim of the WG is to promote the use of NGS across the EURLs' networks, to build capacity within the EU, and to ensure liaison with EFSA and ECDC related to the WGS mandate by the Commission. The WG had its first meeting in Brussels on November 14th 2017 at the Commission in which EFSA and ECDC participated via video link. The WG will focus on the following activities 1) Proficiency Testing, 2) WGS laboratory procedures, 3) Bioinformatics tools, 4) WGS cluster analysis: 5) Benchmarking, 6) Training on NGS, and 7) Reference and confirmatory testing using NGS. Most of the work will include the development of guidance documents and reports on how to target the above-mentioned areas and to avoid redundancy these will include already available resources. Initially, a survey will be launched to provide the overview of the current level of competences at NRL level.