12th EURL-AR Workshop, Kgs. Lyngby, April/2018 – minutes

The minutes are listed according to the agenda.

Participants

From the EURL-AR-network, all member states (MS) were represented at the workshop. Participating non-MS were Albania, Iceland, the former Yugoslav Republic of Macedonia (fYRoM), Norway, and Switzerland. Additionally, representatives from the EU Commission and EFSA participated and also project participants from the CoVetLab-project of WBVR, APHA, SVA and ANSES: “Build a reference database and strain collection of colistin resistant *Enterobacteriaceae* in food-producing animals and food thereof”.

Thursday, April 5th 2018

Welcome (Rene Hendriksen, EURL-AR)

Meet and greet and introduction to the day’s agenda (Rene Hendriksen, EURL-AR)
This year, we have a new contact person at the European Commission; we welcomed Mr. Aurelien Perez, who was recently appointed as the desk officer responsible for the EURL-AR, together with Mr. Martial Plantady. We are looking forward to continuing our fruitful collaboration with the EC.

The agenda for this meeting included discussion of network tasks; e.g. the EQAS organization and results from 2017 as well as a number of other issues related to our area of responsibility.

Update from the EURL-AR (Rene Hendriksen, EURL-AR)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

A key task for the EURL-AR is to give scientific advice and support to the EU Commission. This year, it included updates of the protocols for isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from meat and caecal samples in relation to allowing for 96h storage of the caecal samples with the purpose of having samples from all five working days of the week. Also, a protocol on quantification on ESBL-, AmpC- and carbapenemase-producing *E. coli* from meat and caecal samples and a protocol to perform multiplex PCR for *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* have been published on the EURL-AR website (https://www.eurl-ar.eu/protocols.aspx) .

Moreover, work is ongoing to publish an updated protocol on isolation of MRSA from food-producing animals and farm environment.

The EURL-AR conducted a MIC-reading survey to test the agreement in reading MIC values across the NRL-AR and also conducted confirmatory testing in collaboration with EFSA.

In addition to activities in relation to WHO, in 2017 the EURL-AR has set up PTs within the field of Whole Genome Sequencing (WGS) for the United Nations Secretary-General's Mechanism for Investigation of Alleged Use of Chemical and Biological Weapons (UNSGM).

The EURL-AR also arranged and hosted a training course in November 2017 which aimed at creating milestone plans to setup next generation sequencing for AMR in the MS. Furthermore, we arranged and provided the annual EQAS's within AST, optional genotypic characterization and a matrix EQAS.

Update from the EU Commission (Martial Plantady, European Commission)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

AMR causes deaths and economic loss, and the EC has again passed an AMR action plan (https://ec.europa.eu/health/amr/sites/amr/files/amr_action_plan_2017_en.pdf) based on the recommendations from the previous action plan (2011-2016). The new action plan against AMR (European One Health Action Plan) contains more than 75 actions under 3 pillars. The 3 pillars: 1) Making the EU a Best Practice Region on AMR, 2) Boosting Research Development and Innovation on AMR, and 3) Shaping the Global agenda on AMR.

In addition:

- Directorate F performs Fact-finding missions on prudent use (veterinary medicine) in Member States;
- The EC has initiated review of the EU Decision 2013/652 (EFSA mandate) including also considering use of WGS-methods for the purpose of AMR monitoring;
- In relation to VMP (Veterinary medical products) and MF (Medicated Feeds), trilogue negotiations are ongoing;
- In 2017, at the second G7 Chief Veterinary Officers (CVO) forum, it was agreed on a common approach on the definitions of the therapeutic, responsible and prudent us of antimicrobials in animals.

Update from EFSA (Pierre-Alexandre Beloeil, European Food Safety Authority)

See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

In relation to overall antimicrobial consumption, the JIACRA II report presents considerable variations between countries and between the human and animal sectors. Outliers are detected, however, the report concludes that the data confirms the positive association between antimicrobial consumption and antimicrobial resistance in both humans and food-producing animals and underlines the need to ensure prudent use so as to reduce the consumption of antimicrobials in both food-producing animals and humans.

A few highlights from the report:

- Overall antimicrobial consumption in animals is higher compared to overall antimicrobial consumption in humans;
- Consumption of 3rd and 4th gen. cephalosporins and fluoroquinolones is higher in humans compared to animals, whereas the opposite applies for consumption of colistin;
- There is a positive correlation between AMU (antimicrobial use) and AMR (antimicrobial resistance) for fluoroquinolones and *Escherichia coli* (both invasive and indicator);
- There is no positive correlation between fluoroquinolone use in humans and fluoroquinolone-resistant *Salmonella* sp. in humans, but there is positive correlation in poultry);
- Importance of the indicators for measuring progresses made in implementing action plans against AMR.

EC mandate on the Technical support to collect and analyse WGS data in the joint ECDC-EFSA molecular typing database (by April 2019), and also on drafting the new EFSA Technical specifications on the harmonized monitoring of AMR in bacteria transmitted through food (by March 2019).

Outcomes of the EURL-AR EQAS 2017

The three EURL-AR EQAS reports were approved without further comments:

- EURL-AR EQAS *E. coli*, enterococci, staphylococci 2017 report
- EURL-AR EQAS Matrix 2017 report
- EURL-AR EQAS *Salmonella*/*Campylobacter*/genotypic characterization 2017 report

Additionally, summary on the outcome from discussion in groups is collected in Appendix 1.

The CoVetLab project (Dik Mevius, NL)

See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

CoVetLab is an active collaboration between five reference institutes (APHA, DTU, SVA, ANSES and WBVR). Annually it stimulates small to medium collaborative studies between CoVetLab partners. The CoVetLab project presented at the meeting was set up to build a reference database and strain collection of colistin-resistant *Enterobacteriaceae* in food-producing animals and meat thereof.

An overview of the *mcr* genes' history was given as regards discovery of the genes conferring colistin resistance and as regards the evolutionary steps involved in the spread of the genes. Currently, *mcr* genes have been detected globally. The occurrence of the *mcr* genes is associated to colistin use (and intervention by limiting colistin use is effective in reducing prevalence of *mcr*), however, the public health risks linked to occurrence of *mcr* in animal isolates is debatable.

Break-out groups focused at *mcr* and NGS based on the experience from CoVetLab

Question 1.

a. How do you detect phenotypic colistin resistance in *E. coli/Salmonella*?
b. What is the preferred phenotypic method?

Question 2. Which method do you use to identify *mcr* genes?

Question 3. Is additional characterization performed (genes/IS elements, plasmids, strains)

Question 4. How much data is available on *mcr* positive isolates and what is the origin of the isolates?

Question 5. Are there restrictions in sharing data?

Question 6. Is *mcr* a public health threat? And if so what actions have been undertaken?

Question 7. Do we need to improve the EU-monitoring activities?

Re question 1:
Methods mentioned were agar dilution and the method described by Patrick Butaye (pre-diffusion test with Rosco tablets).Dik mentioned that for surveillance purposes, the EUCAST method is preferable.

Re question 2:
Methods mentioned were PCR and WGS. Isolation of colistin resistant isolates is done on MacConkey with 1 – 2 mg/L colistin.
Dik mentioned that this will be elaborated further in the current European project, IMPART (EJP).
Biomerieux is producing chromogenic agar plates to identify colistin-resistant isolates.

Re question 3:
In one breakout group, 3/6 used the multiplex PCR screening by the EURL-AR (Rebelo et al., 2018) on isolates.

Re question 4:
Ranged from no confirmed *mcr*-positive isolates to up to 700 detected colistin-resistant isolates.

Re question 5:
CoVetLab experienced a high degree of willingness to share data.

Re question 6:
In low-prevalence-countries it is not considered as a public health threat.

Re question 7:
May be included in national country programmes (integrated between human and veterinary health), it really depends on the country prevalence of mcr.

ISO standard on WGS (Errol Strain, US Food and Drug Administration)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

The draft standard (TC34/SC9/WG25 Genomic sequencing of foodborne microorganisms – General requirements and guidance for bacterial genomes) has been made by representatives from a number of countries.

A process is defined for drafting and finalizing ISO-standards, and the current draft is still very early in the process, consequently, the content presented today may change compared to the document that will be published in due time. It is important to underline that the ISO process is long and requires 2-3 years at least.

The draft includes three sections: 1) Laboratory operations (DNA extraction and isolate to sequence data), 2) Bioinformatic pipelines (functional and/or cluster predictions), 3) Metadata (enabling traceback, surveillance, data sharing).

Quality assessments are necessary when using this method for analysis. The reason for not including precise quality parameters such as coverage and other quality threshold is that the ISO should be useful over a relatively long time. As quality parameters are very specific according to sequencing platforms, if they are included the ISO standard will not be universally valid for long. Thus, the quality parameters included in the ISO must be non-specific to the sequence platform. For data analysis, most users of the standard may not have their own pipeline, the idea is therefore that the standard describes how to validate the correct installation of the pipeline rather than describing the full validation of a pipeline.

An example of pipeline validated for AMR surveillance purposes is that established by Patrick McDermott for Salmonella susceptibility testing towards 14-15 antimicrobials: they observed 99% correlation with phenotypic results but this was tailored on their purposes and it does not necessarily mean it is universally valid. They also provide real-time availability of AMR surveillance data through the NCBI pathogen detection.

The standard is intended for food microbiology and it is important to capture metadata together with sequence data. The intention is to capture relevant metadata so that it is useful to the laboratories and not a burden.

Summary of the plenary discussion:

Errol recognized that functional predictions about isolates are important and remarked that even though there may not be an easy solution, validation of the applied method is important.

Errol mentioned in the presentation that the system should be 'fit for purpose'. If working in a reference laboratory some decisions must be made. Errol stated that much is left up to the user. The ISO standard cannot specify all thresholds as they would be defined based on the platform and also as the standard needs to be useful for years after it has been published. For example, if a sequencer can sequence 100% correctly, the standard should also be working for that type of sequencer.

Asked to comment on in-house pipelines vs. validated pipelines, Errol commented that likely, individual laboratories' bioinformaticians time resources are better used than setting up in-house pipelines. Many in-house pipelines are also probably not published.

The transition to WGS for monitoring is on the way, currently the Center for Veterinary Medicine (CVM) at FDA in the US is screening using 'resistome-trakr' (https://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm570694.htm)

The outcome of the EFSA *Campylobacter jejuni* project GENCAMP using WGS – fluoroquinolone-resistance (Pimlapas Leekitcharoenphon (Shinny), DTU Food)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

In the GENCAMP project, a high genomic diversity was observed when analyzing quinolone-resistant and susceptible *Campylobacter jejuni* of poultry origin from major poultry producing European countries. Multiple ST types and also a large number of SNPs were observed.

The different phylogenetic methods provided concordant results and all phylogenetic trees revealed that the isolates clustered according to the presence/absence of *gyrA* mutation and ST complex.

None of the phylogeny methods can cluster strains based on the country of origin.

The allele frequency indicated the association with geographical origin of the poultry *C. jejuni* isolates, thus analysis of allele frequency would be an alternative genomic approach for identification of geographical origin and source attribution of *C. jejuni*.

Based on the data available and the analysis performed in this study it cannot be concluded whether this relates to independent selection within countries or transmission of already resistant clones between countries.

Summary of the plenary discussion:

It was observed that a *Campylobacter* that enters into another host will change to a different ST. It is diverse, and back-tracking cannot be done.

For the applied setup, assessing the cgMLST, 1300 loci must correspond. Potentially this method resolution might be too high for this purpose, the discriminatory power could be lowered by lowering the cutoff. Also it would be an option to analyze a conserved part of the genes only, or the essential genes rather than the core genes.

The fact that the same topology was observed with different approaches is interesting. Follow-up analyses to investigate the *gyrA* further will be performed by setting up machine learning.

The EURL working group on NGS (Rene Hendriksen, EURL-AR)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

The EU Commission has established a working group for the purpose of promoting the use of NGS across the EURL networks. The WG-tasks have been defined and responsibilities divided between the EURLs.

The outcome of the WG will include producing and publishing protocols and guidelines, tutorials and E-learning and also, a proficiency test will be setup to measure the quality of the sequencing in the European laboratories.

The WG is conscious that the work related to the ISO standard is important and necessary, even if it is resource demanding.

Other projects are ongoing with a focus on WGS and the collection and sharing of data.

E.g., the EC, ECDC and COMPARE are working on setting up genomic surveillance of *Salmonella*, with nine laboratories currently involved.

The legal aspects still need to be finally defined.

The incentive of sharing genomic data through a EURL-AR hub is that instantly when you share your data, you also receive analysis of your sequence. The data hub (private) is part of the Short Read Archive at ENA.

Further info on this private data hub will be forwarded to the EURL-AR network by the EURL-AR.

Also, a global sewage project is currently ongoing. As part of this project, DTU Food collected sewage from all over the world and has been working to detect AMR genes in the sewage. The first draft of a publication is ready.

The next step is that we are looking into waste water from slaughterhouses. We strive towards getting a uniform sample that represents all animals slaughtered at the slaughterhouse. We welcome participants from all countries and will send out an invitation with further information to the EURL-AR network.

Friday, April 6th 2018

Transposable elements and their role in the spread of antimicrobial resistance in bacteria (Adam Roberts, Liverpool School of Tropical Medicine, UK)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

Transposable elements are segments of DNA able to excise from a replicon and re-insert. Likely, there are consequences of the jump of the transposon – both due to the excision and due to the insert (in a gene or between genes). Also, transposable elements can usually invert.

Insertion sequences are responsible for the mobilization of many genes which flank their insertion sites. Once mobilized onto a composite transposon, and challenged with a suitable selective pressure, they will facilitate the transfer into multiple replicons.

Potentially, AMR surveillance programmes should include a survey of the agents of dissemination (ISs) as well as antimicrobial resistance genes.

Functional metagenomic analysis for discovery of novel antimicrobial resistance genes can give an indication into what might emerge given a new selective landscape.

Summary of the plenary discussion:

Definitely, additional characterization as regards genes/IS-elements, plasmids, strains should be performed if the data and resources are available.

The relevance of performing metagenomics analyses of gut microbiota, soil and oral microbiota was discussed. Adam considered it a funding issue that no more screening was done and mentioned that it would be beneficial to screen samples of soil using metagenomics.

Some libraries will not give you the transposable element because of the short reads. Adam recommended PacBio (if you have a lot of money), otherwise combining Illumina data with Nanopore data and use MinION Nanopore library will work.

Outcome of the EFSA reference testing; confirmatory testing (Rene Hendriksen, EURL-AR)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

The annual reference testing is a collaborative work between the EURL-AR and EFSA. EFSA selects the relevant strains based on the AMR surveillance data submitted by each MS and following specific criteria. At the EURL-AR, the MIC determination is repeated and WGS is performed including use of ResFinder 3.0 (detecting AMR by chromosomal mutations and de facto AMR genes). These results are compared and reported back to EFSA and to the relevant NRL-AR.

Mostly, we observe a nice concordance between the genotype and phenotype. For azithromycin there may currently be some gaps, though.

Please pay close attention to this very important message: Strain management is crucial. Please keep all strains for later reference in case situations occur in which we need to follow-up.

ResFinder v.4 update (Valeria Bortolaia, EURL-AR)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

ResFinder 4.0 is a bioinformatics analysis tool intended to produce *in silico* antibiograms. It consists of a database of AMR genes including *de facto* AMR genes (i.e. whether the gene actually is 'acquired' or 'intrinsic' depends on the microorganism in question) and chromosomal mutations leading to resistance (e.g. *ampC* promoter mutations, *gyr*A mutations, etc.).

If no AMR gene is detected, this does not necessarily mean the isolate exhibits no antimicrobial resistance (unknown genetic mechanisms may be present). Also, even if an AMR gene is detected, this does not necessarily mean that the isolate exhibits antimicrobial resistance (e.g. a gene might not be expressed, it may have mutations leading to stop codons, etc.).

Neither phenotypic nor genotypic methods are perfect for AMR prediction. The main limitation of *in silico* antibiograms is our incomplete knowledge of genetic basis of resistance.

Summary of the plenary discussion:

Concordance between genotypic and phenotypic has not yet been tested. Currently, we have MIC-data and WGS sequences (and isolates in the freezer) and the idea is to verify the concordance species by species and provide an output only for the antimicrobials relevant for the microorganism in question.

The EURL multiplex protocol on *mcr-1, -2, -3 -4, -5* (Ana Rita Rebelo, DTU Food)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

Phenotypic testing of colistin resistance must be done by broth microdilution, though some problems are still observed with reproducibility of MIC results.

The EURL-AR multiplex protocol on *mcr-1*, *-2*, *-3, -4*, *-5* is fast (< 24h) to run. It is, however, advised to perform MIC-determination on the strains first (to detect resistance) and then subsequently detect the gene using PCR, considering that some isolates might harbour other mechanisms of colistin resistance.

Between the development of the protocol an the presentation two new *mcr* genes were described: *mcr-6* and *mcr-7. In silico* analysis shows that the current primers for *mcr-1*, *-2*, *-3, -4*, *-5* do not anneal to *mcr-6* and *mcr-7* and new primers will be designed and added to the protocol.

Summary of the plenary discussion:

An option could also be to run the multiplex PCR on DNA directly extracted from the sample, which is routinely done in the Netherlands followed by culture of positive samples. This has however not been tested at the EURL-AR.

The real-time *mcr* PCR by ANSES (Hattie Webb, ANSES)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

The work with *mcr*-positive strains in France was based on 8,684 strains. The French *Salmonella* Network used a disk-plate including also a colistin disk for control purposes. It was observed that the zones around some of the colistin disks were slightly smaller. This lead to the detection of five truly colistin-resistant isolates of different serotypes and originating from different parts of France.

For *mcr-2, -3, -4* and *-5*, new primers were designed and we setup a real-time PCR to look for *mcr* genes in the monitoring programme.

Results from the MIC survey 2017 (Jette Sejer Kjeldgaard, DTU Food)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

A survey consisting of 45 photos of MIC-panels was circulated and it was suggested to read only the panels inoculated with the microrganisms normally tested in the laboratory.

For analysis of the results, two rules were decided 1) “Mostly selected MIC” was defined as the expected result, 2) Results were classified as ‘acceptable’, ‘unclear MIC’ and ‘unclear resistance’

Note that

- For beta-lactams – any growth (independent of the size) should be considered as growth.
- For sulfonamides, it is important to know that due to antagonists in the media the MIC should be defined at the well where there is 80% less growth compared to that of the positive control.

It appears that most deviations in MIC reading occur when there is poor growth in general and for bacteriostatic antimicrobials (e.g. sulfonamides).

Summary of the plenary discussion:

One NRL commented that if one skip is observed on a plate and that does not cause a change of interpretation of a result from S to R, re-test is not performed. If it changes the interpretation, re-test is performed.

Country presentation: Activities of the NRL-AR in Macedonia (Sandra Mojsova, NRL Macedonia)

See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

The NRL-AR in Macedonia is based at the Faculty of Veterinary Medicine in Skopje.

As regards to antimicrobial resistance, the laboratory staff was trained by an expert from Italy. The laboratory is working with an SOP based on ISO 20776-1.

The NRL-AR is planning on participating to the EURL-AR EQAS 2018.

Summary of the plenary discussion:

Till now, the isolates we have analyzed have been relevant for projects. Next year, we will be analyzing isolates relevant for monitoring.

Country presentation: Experience with the EURL-AR protocol in a field study of VIM-mediated resistance in Germany (Mirjam Grobbel, NRL Germany)

See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

As part of a project to look into the spread and persistence of carbapenemase-producing *E. coli,* attempts to trace back the VIM-positive strain detected late 2015 were done. In March 2016, on short notice, samples were received and it was not possible to receive “carba plates” in the short time span. Therefore, different attempts were set up to not miss possible VIM-1 producers, i.e. direct plating and selective enrichment followed by plating.

Samples from the piglet truck (before they entered the farm) were found negative.

For samples from the six barns, the primary approach lead to one VIM-positive isolate. The rest of the plates showed overgrowth of background flora.

Subculturing back and forth lead to detecting four VIM-positive isolates

A non-selective enrichment and real time PCR were then performed. We observed that even slight signals appeared to indicate the presence of a VIM-positive isolate.

Summary of the plenary discussion:

Samples from pig-breeders were also taken and the analysis is ongoing.

In Germany, in fact, since some years, the whole production chain has been tested each year for different bacteria. In the state laboratories they sample farms.

The current EURL-AR protocol is setup to serve multiple purposes. We, at the EURL-AR, will take this as an opportunity to revisit the protocol (as part of the EJP project, IMPART, and also at the EURL-AR).

Update on protocols on isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from meat and caecal samples (Valeria Bortolaia, EURL-AR)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

In 2017, the protocol for isolation of ESBL-, AmpC- and carbapenemase-producing *E. coli* from meat and caecal samples was updated with information that the window of sample storage could be extended to 96h. The decision to extend the window of sampling storage was not to keep all samples for 96 hours, but to allow for sampling on Thursday/Friday.

In the case of meat we have an 'easier' situation, as the analysis must be started at the latest on the expiration date or same-day.

With regard to media for selective isolation, commercially available chromogenic agars are recommended.

Also, a subcultivation step has been introduced. ESBL/AmpC-positive strains should be subcultured on MacConkey+CTX. Carbapenemase-positive strains should be subcultured on the commercially available plates (or, if not available due to funding, on a MacConkey (with no CTX-addition!) agar plate.

Summary of plenary discussion:

Some use the same caecal sample for indicator *E. coli* and also for *Campylobacter*. This protocol, however, is relevant for the *E. coli*, only, as the mandate of the EURL-AR is related to the ESBL-positive *E. coli*.

Iceland has an issue related to frozen meat. Whether this protocol can be applied to meat that has been frozen for 30 days (which is done for all imported meat to Iceland – frozen in 30 days, defrosted and sold as fresh) is a peculiarity related to Iceland and will currently not be considered a priority for the discussions.

The protocol describes an incubation temperature for the *E. coli* at 44ºC. Some *E. coli* will not grow at 44ºC, and it was stated that we may miss some especially from ruminants and pigs. Also, it was also stated that at lower temperatures false positives may be seen (which will then be ruled out during the confirmation step). The issue of incubation temperature will be taken into consideration when setting up the validation study at the EURL-AR later this year.

Also, in the validation it will be taken into consideration whether the selective plates may be stored after the pre-enrichment over the weekend so that the analysis may be continued on Monday (this approach has shown to work well in research projects).

Protocol on quantification on ESBL-, AmpC- and carbapenemase-producing *E. coli* from meat and caecal samples (Valeria Bortolaia, EURL-AR)
See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

According to 2013/652/EU it is voluntary to perform the quantification of the ESBL-, AmpC- and carbapenemase-producing *E. coli* from meat and caecal samples. The protocol is particularly useful when there is a high number of ESBL-producing *E. coli*. The protocol is not intended for counting carbapenemase-producing *E. coli* (it is likely that they are currently occurring, if present, below the detection level).

Incubation at 44ºC was selected to be consistent with the other ESBL/AmpC-protocols.

Summary of the plenum discussion:

The ISO standard in relation to enumeration of *E. coli* uses TBX. TBX was originally also included in the tests at the EURL-AR in the preparatory work to make this protocol but this choice was for some reason abandoned. We will follow up on the reason for that.

It was suggested to use also *Citrobacter* when spiking samples as *E. coli* and *Citrobacter* have a similar appearance on MacConkey agar plates.

Update on protocol on isolation of MRSA from dust samples (Valeria Bortolaia, EURL-AR)

See presentation (link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2018.aspx)

The protocol for isolation of MRSA from dust samples will be updated to describe a cheaper and more sensitive method that does not include a selective enrichment step.

The protocol is being finalized and will be available on the website (https://www.eurl-ar.eu/protocols.aspx) a.s.a.p.

Summary of plenum discussion:

- The publication describes nasal swabs. Dust and nasal swabs seems to be quite different. Info on the difference between the two types of samples can be retrieved from the authors.
- Similar studies from the NRLs (with fresh pork meat) also found that the one-step enrichment method was more sensitive than the two-step method.
- It was commented that with *mecC* MRSA it appears that additional cefoxitin will severely lower the detection rate.
- A project on dust samples using gloves is ongoing in the Netherlands.

AOB and plenum discussion

The EURL-AR network was invited to bring up issues for discussion!

Remember that the following invitations will be sent to you:

- Invitation to participate in the CoVetLab project
- Invitation to participate in the Global Sewage project (slaughterhouses)

Note also that at EUCAST it has been decided to use the term ‘Enterobacteriales’ instead of ‘Enterobacteriaceae’ – this is relevant for our legislation.

Future perspective and closing remarks (René Hendriksen, EURL-AR)

Any suggestions for issues to address in future EURL-AR workshops are welcome. Please send them by email to us (rshe@food.dtu.dk).

Next year, in April 2019, we will meet at a joint meeting with the FWD-network.

Appendix 1
Summary from group discussions at the EURL-AR workshop 2018

Experiences and challenges in relation to the 2017 EURL-AR EQAS

In break-out groups, experiences and challenges encountered following the 2017 proficiency tests were discussed. The workshop participants had the opportunity to bring up any important observations with regard to the 2017 EQAS's. This could be challenges faced or other observations made during the testing, or it could be remarks to the draft reports.

Discussion items were been drafted for the groups to consider. All NRL participants were encouraged to read and discuss the following topics/questions locally prior to attending the workshop, and also to bring additional observations, challenges or questions into the discussions.

Following the breakout group discussions, Rene Hendriksen (EURL-AR) headed the plenum summary and discussions, and with this appendix, the main issues addressed by the groups are reported.

Regarding EURL-AR EQAS *E. coli*, enterococci, staphylococci 2017:

1. In the *E. coli* trial, the main issue for the laboratory presenting the highest percentage of deviations (11.7%) was represented by a switch of two strains. Although it is reassuring to notice that there are no issues in performing the AST method itself, strain/sample management issues are very serious and they appear every year (though at different laboratories). What suggestions can be given to avoid these problems? Do you have standard procedures in your laboratory to avoid these problems?

   Response and comments from the breakout groups:

   - Switch of strains is probably not a very common problem.
   - Many laboratories also use the EQAS samples for training of staff, some for example perform the test twice independently by two persons – all the way from subculture to interpretation. Other laboratories have decided to run the EQAS samples as normal testing to represent the real-life situation.
   - Purity control also is important. In this context it was mentioned that some laboratories test two different cultures on the same purity control plate which they argued was not acceptable.
   - Document what is done – at which time and by which persons.
   - If performing molecular typing, use the initial culture received from the EQAS-provider.
   - Human errors in relation to entering results in the database are very common. This is also to be considered a very important step.
   - It is helpful to perform WGS of the strain and match the results with the phenotype.

- It was strongly suggested to keep the stab cultures in the fridge until the final EQAS results are available. In this case,if something is wrong, it will be possible – to retrieve the original culture for further tests.

2. Most laboratories obtained less than 5% deviations from expected results in the EURL-AR EQAS *E. coli*, enterococci, staphylococci 2017 trial, thus showing a very satisfactory performance. The 5% threshold for acceptable laboratory performance was established a long time ago before the harmonisation of AST methods across NRLs. Do you think this threshold should be changed and, for example, set at 3%? (In this case, only true performance problems should be taken into account thus excluding all "one-fold dilution issues" that become problematic when expected results are close to breakpoint). Please explain your answer. Do you have a 5% threshold for acceptable laboratory performance also with your national accreditation body, or do you have a different value? If different, what are the reasons for that?

   Response and comments from the breakout groups:

   - Each laboratory aims at reaching 0% deviation. Setting the limit lower than 5% is not important since the acceptable deviation level is not used an incentive to improve the performance but it is used to explain results to quality managers. It is important to react on all deviations, i.e. follow up on them and explain what caused them, e.g. in a short report explaining the outcome of the EQAS and commenting on any deviations.
   - By keeping the 5% deviation threshold, it will also be possible to follow temporal trends. It is also important to highlight the different error types, for example errors in detection of carbapenemases could be more relevant than other errors.
   - In clinical testing, there is an established way to classify deviations such as 'very major', 'major', 'minor' errors. It could be considered to use this classification also in relation to the EURLAR EQAS.

3. Do you perform follow-up when you receive the EQAS results? If so, what do you do? Do you have standard follow-up procedures? Who is involved in the follow-up (manager, technical staff, etc.)?

   Response and comments from the breakout groups:

   - Follow-up is performed as the EURL-AR EQAS is used for accreditation. Generally the involved personnel discuss the obtained results. It is a bit problematic that the final report is available only months after the EQAS ended. However, there is no real solution to this problem since the report has to be approved by the entire network and the only possibility for that is at the annual EURL-AR workshop.

Regarding EURL-AR EQAS Matrix 2017:

4. It seems there are (still) problems with contamination/unspecific growth on both MacConkey/cefotaxime and carba plates. Discuss your findings and how to improve the protocol. Did you try other protocols for the samples in parallel?

Response and comments from the breakout groups:

- Many laboratories encountered one-step-dilution issues when performing the AST.
- In some countries, a suspected colony is streaked a second time on a selective plate for the purpose of obtaining a pure culture.
- Variable organisms also grow on some types of MacConkey agar plates. A suggested solution for this could be multiple testing at the EURL-AR and that the receiving laboratory performs WGS.
- As for Carba plates, ensure that the streaking of plates is done in accordance with the method protocol. Streaking is an essential stage to maximize the possibility that the inoculum allows recovery of the target organism if it is present, without being obscured by overgrowth of contaminants/non-target organisms.
- The expiry date of the plates is very important – you might still get relevant results from expired plates, though if using expired plates for the testing, validation of their performance must be performed.
- A suggestion for future Matrix EQAS's could be to have information about the background flora, i.e. the EURL-AR could introduce a metagenomics analysis of the sample in the preparatory work.

5. Do you pretreat the caecal samples before enrichment? Some has implemented a washing step to remove bacteria on the outside of the caecal – others use the caecal content directly. Discuss your approach and the pro/cons.

Response and comments from the breakout groups:

- It was highlighted that it is the caecal *content* that should be tested – not the total caeca as this will introduce a large bias (the caecum is highly subjected to contamination at the abattoir).
- Different approaches were mentioned, i.e. washing/no washing of the caeca, spraying the caeca with 70% ethanol/no spraying with 70% ethanol. There was no information as to there is a difference in results between washing/not washing or spraying/not spraying.
- Discussion of practice when pooling the samples; in some countries, one caecum is tested, other countries pool two, three, five caeca or up to 10 caeca. Most countries pool 10 caeca from broiler chickens as this is written in the Decision. The different pooling bring analytical and diagnostic sensitivity differences, but the effect is different based on the occurrence of the target bacteria.
- Some laboratories use the collected caeca for indicator *E. coli* and also for the voluntary testing of *Salmonella.* Regarding contamination of samples in relation to isolation of *Campylobacter*, the EURL *Campylobacter* network was informed to spray with 70% ethanol.

Regarding EURL-AR EQAS *Salmonella*/*Campylobacter*/genotypic characterization 2017:

6. The *Salmonella* EQAS showed examples of participants encountering an unexpected phenotype when performing AST of a test strain – in particular this was the case with regard to test strain S-12.4 (AmpC-producer and at the same time ampicillin-susceptible). In

general, how does your laboratory handle unexpected phenotypes? Discuss which procedures are relevant to have in place to detect and confirm an unexpected phenotype which might be a novel and potentially emerging.

Response and comments from the breakout groups:

- Most laboratories have a protocol on what to do when an unexpected antimicrobial resistance profile is seen. A summary of the methods is that firstly, re-test is performed, and then different additional testing using a different phenotypic approach (e.g. E-test). Then, some perform PCR, WGS or microarray and go through the EUCAST guidelines and other guidelines.
- There is a difference between screening breakpoints and epidemiological cut-off values for cefotaxime and ceftazidime and this sometimes causes problems for the reporting. The EURL-AR will discuss this issue with EFSA and forward the conclusions to the EURL-AR network.

Comment from the EURL-AR: If you do not have local guidelines for unexpected phenotypes, see the one developed by the EURL-AR (available on https://www.eurl-ar.eu/resources.aspx). Moreover, you are always welcome to contact the EURL-AR for input and guidance.

7. The test strain S-12.4 presents an unusual phenotype (AmpC-producer and at the same time ampicillin-susceptible, unknown resistance mechanism). Has this phenotype been detected in *Enterobacteriaceae* in your country?

Response and comments from the breakout groups:

- Very few countries could say ‘yes’ to this question. Most responded ‘maybe’.
- An unusual phenotype observed in clinical diagnostics was cefepime R and cefotaxime S
- In fish products, they found IMI in Enterobacter conferring an unusual phenotype

8. In the *Campylobacter* trial, the analysis of the data revealed that C-12.1 was probably a mixed culture. The strain was expected to be fully susceptible, however, six laboratories reported resistance to ciprofloxacin, nalidixic acid and tetracycline based on high MIC-values not close to the breakpoint. Consequently, it was decided to exclude all the results of the six laboratories in question in relation to C-12.1. This is an example of a situation in which the laboratory might have reported a results from a correctly performed AST but which was subsequently unjustly evaluated as incorrect.
   a. When participating in an EQAS, which level of deviations is accepted at your laboratory? At which level of deviation is troubleshooting and follow-up initiated?
   b. At your laboratory, when experiencing that results you submitted for an EQAS were evaluated as incorrect, how does your laboratory react on this to identify what may have caused this discrepancy between the obtained and the expected result? Discuss which procedures are relevant to have in place to perform the sufficient troubleshooting to detect the cause and to set up measures to ensure that the mistake does not happen again.
   c. Discuss how participating in an EQAS is useful to your laboratory.

Response and comments from the breakout groups:

Issues in relation to the above questions were overall discussed in the previous questions also.

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