13th EURL-AR Workshop, Kgs. Lyngby, 25-26 April/2019 – minutes

The minutes are listed according to the agenda.

Participants

From the EURL-AR-network, all EU member states (MS) were represented at the workshop except for Cyprus, Malta and Finland. Participating non-MS were Albania, Iceland, North Macedonia, Norway, and Switzerland. Additionally, EFSA was represented with a workshop participant.

Three researchers from Ghana (funded by the Fleming Fund) and two from Thailand (funded by FAO/IAEA) participated as observers as did also a representative from Federation of Veterinarians of Europe (FVE).

Thursday, April 25th 2019

Welcome (Rene Hendriksen, EURL-AR)

Meet and greet and introduction to the day's agenda (Rene Hendriksen, EURL-AR)
The agenda for this meeting included discussion of network tasks; e.g. the EQAS organization and results from 2018 as well as a number of other issues related to our area of responsibility, this year, to a large degree focusing at Whole Genome Sequencing (WGS).

Update from the EURL-AR (Rene Hendriksen, EURL-AR)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

A key task for the EURL-AR is to give scientific advice and support to the EU Commission. This year, it included validation of the 96h storage of the caecal samples with the purpose of analysing samples from all five working days of the week (document available for download from https://www.eurl-ar.eu/resources.aspx).

The EURL-AR arranged and hosted a training course in September 2018 which aimed at applying WGS of bacterial isolates for AMR-monitoring in the MS. Furthermore, we arranged and provided the annual EQAS's within AST, optional genotypic characterization and an EQAS

on selective isolation of *E. coli* with presumptive ESBL or AmpC phenotypes from meat or caecal samples (matrix EQAS).

For the 2019 EURL-AR EQAS, a new database is under development. This new database will be available for submission and evaluation of the EQAS results as of this year. It will allow for an easily accessible output of results overview (pdf-version).

One of the large tasks for the EURL-AR in 2018 has been the EFSA/EURL confirmatory testing. For 500 isolates we performed phenotypic and genotypic antimicrobial susceptibility testing followed by comparison and analysis of the obtained results.

The EURL-AR have been represented in the EFSA working group working towards the new EU decision planned for 2021.

The EURL-AR have collections of reference material which the network are welcome to look into. Should an NRL be interested in receiving a subculture of a specific reference, please contact the EURL-AR directly.

We encourage all NRL's to contact the EURL-AR directly as regards any changes in the contact persons related to their laboratory.

Update from EFSA (Pierre-Alexandre Beloeil, European Food Safety Authority)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

For the *Salmonella* spp. the number of isolates varied importantly between countries, i.e. the dataset was not easy to analyse. It was observed that the levels of resistance was typically lower in *Salmonella* spp. From calves compared to those from pigs. The key indicators were 1) combined resistance to critical antimicrobials in *Salmonella* from humans (CIP and FOT) which was high in Malta (12.6%) and Poland (8.6%), and 2) MDR resistance which was considerably higher in *Salmonella* spp. from pig carcasses than calf carcasses.

For *Campylobacter coli* and *C. jejuni* in poultry, high levels of resistance to fluoroquinolones were observed. There was, however, high variability between member states. As for the combined resistance to critical antimicrobials in *Campylobacter* from humans (CIP and ERY), a few countries reported high (4) or very high (1) combined resistance in *C. coli* (20.1-59.5%).

For the indicator *E. coli*, high resistance levels were observed to commonly used antimicrobials. For third generation cephalosporins and macrolides, resistance was reported by some MS, though at a very low level. As regards complete susceptibility (susceptible to all tested antimicrobials), a north-south gradient can be observed.

Following AMR in indicator *E. coli* from fattening pigs long term, indicates decreasing trends in tetracycline. As this is a widely used antimicrobial, this would be connected to other resistances also, i.e. likely this indicates a decrease in the complete panel of antimicrobials used. Trends in complete susceptibility in *E. coli* from pigs will be followed in the years to come.

The specific monitoring of ESBL- and AmpC-producing *E. coli* indicates that the prevalence is much higher in animals compared to the prevalence in pig meat and bovine meat and there are higher levels of ESBL-producers than AmpC-producers, reflecting the prevalence over the food chain.

As for MRSA, it is being discussed that potentially a new baseline study should be launched.

A third JIACRA report is planned by ECDC, EMA and EFSA to be produced by the end of 2020.

Summary of the plenary discussion:

The map of pan-susceptible *E. coli* is interesting. The indicator should increase if there is less antimicrobial consumption. The EU Commission can set up further actions in the action plan for example to improve the sanitary state of the herds.

Update from the EU Commission – European One Health Action Plan against Antimicrobial Resistance (Aurelién Perez, European Commission)

The European Parliament adopted in September 2018 a resolution on the European One Health action plan against AMR. Member States are also working on antimicrobial resistance, e.g. a ministerial conference on antimicrobial resistance will be organized in The Netherlands in June 2019 and Council conclusions are being prepared on the next steps towards making the EU a 'best practice region' in combatting AMR.

As for the regulatory aspects, new EU legislation on veterinary medicines and medicated feed was adopted in December 2018.

In March 2019 a Communication came from the Commission on the strategic approach to pharmaceuticals in the environment. In relation to stakeholders, two meetings of the AMR One Health Network have been organized during the last year.

The Better Training for Safer Food initiative on AMR is now available also in non-EU countries also, e.g. Argentina.

As for international relations, activities are ongoing, e.g. G7/G20, also cooperation with WHO and OECD as well as activities with enlargement and neighborhood countries.

All the details of the implementation of the 2017 AMR EU Action Plan (https://ec.europa.eu/health/amr/sites/amr/files/amr_action_plan_2017_en.pdf) are presented in regular progress reports (http://ec.europa.eu/health/amr/sites/amr/files/amr_2018-2022_actionplan_progressreport_en.pdf).

EFSA/EURL-AR Confirmatory testing (Beatriz Guerra Román, EFSA, and Valeria Bortolaia, EURL-AR)
See presentation (direct link (Beatriz), direct link (Valeria) or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

Beatriz Guerra Román:
The EFSA/EURL confirmatory testing applies WGS to support the AMR surveillance. EFSA selects the isolates for testing and a flow of tasks is set in motion to confirm the AST results.

The selection of the isolates for the 2018 exercise was based on carbapenem-resistance, ESBLs and AmpC, any non-solved discrepancies between P1 and P2 (more than one level of difference between P1 and P2 or in the case of meropenem, one level difference) as well as some selected antimicrobials like tigecycline, colistin, and azithromycin.

The process of the confirmatory testing starts in June. MIC re-testing is performed so that the MSs can report EFSA any changes in November the latest. Comparison of the WGS results goes into 2nd/3rd quarter of the following year.

Valeria Bortolaia:
In the confirmatory testing related to AST results from 2017, contamination and species identification issues were observed.

For the dataset of 10,080 strain/antimicrobial combinations obtained, phenotype-genotype concordance was observed in 98.5% results. The observed phenotype-genotype discordances (121 cases of phenotypic resistance but no gene/mutation detected and 30 cases of phenotypic susceptibility in presence of gene/mutations) may be due to i) incomplete database of AMR genes, ii) need to set search parameters different from the default ones, iii) incomplete knowledge of AMR mechanisms, and iv) gene expression issues.

Notably, there were 10% of the isolates showing phenotype-genotype discordance for azithromycin. Another notable isolates was a meropenem-resistant *E. coli* which however did not appear to produce any carbapenemase by the carbapenem inactivation method (CIM test) and did not yield any carbapenemase-encoding gene according to ResFinder and CARD analysis of WGS data.

Additional observations that could be drawn from a preliminary analysis of the WGS data were:
- The circulating pool of ESBL-AmpC genes and PMQR genes is quite broad, with some variants detected in several countries and other variants that appear to be localized in a

restricted number of countries
- The phylogeny of *Salmonella* Rissen isolates was constructed to understand if some specific clones were circulating in EU countries. Overall, it does not seem that there is a clonal spread across countries.

Summary of the plenary discussion:

The next step for the meropenem-resistant isolate will be to look into porin modifications which could explain the ertapenem resistance but still would leave the meropenem resistance partially unexplained. In any case, as the isolate was found resistant by the MS but not confirmed at the EURL-AR, it will be left out of the EUSR report.

For the cases in which the mph(A) gene was detected in azithromycin-susceptible isolates, the fact that mph(A) is part of an operon and mph(A) expression is regulated by genes in this operon was mentioned as a potential reason for the observed phenotype-genotype discordance. Analysis of the entire operon could be the next step to try to solve the phenotype-genotype discordance.

The fact that the timeline stretches from June one year to the 3rd quarter the following year was discussed. All the communication involved takes time, also taking into consideration the holiday periods over summer. All isolates arriving at the EURL-AR are cultured and subcultured to ensure purity and to ensure that we are testing the same strain that the MS tested and sent. Pierre-Alexandre, EFSA, stated that fortunately, more and more MS are now able to deliver a more validated dataset with less discordances. We would hope to speed up the process. By May 2019 we will send out information in relation to the timeline for the upcoming confirmatory testing.

The EURL-AR asked the NRL's if they had looked into the cases in which phenotypic resistance was detected but no gene was found. One laboratory mentioned that they found genes in two ESBL-producing isolates in which no genes were detected by the EURL-AR. Valeria follows up with this laboratory to investigate.

EFSA Scientific Report on the review of the AMR monitoring (Pierre-Alexandre Beloeil, EFSA)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

The intention is that the new EU decision is implemented over the period 2021-2026.

The terms of reference of the mandate for the EFSA WG is to review and update when necessary the EFSA Technical Specifications taking into account new scientific developments as to recent trends in AMR. When including and applying molecular methods, we need to take

into account that it is necessary to be able to compare to the previous method applied (Broth Microdilution).

Views and direct feedback from the MS was requested in a survey which collected information on isolation of *Campylobacter* as well as various specific issues in relation to AMR.

As for the review of the EFSA Technical Specifications, the frequency of sampling on a biennial basis is acceptable.

Changes proposed for monitoring of *Campylobacter* include slight alterations of the harmonized panel, and to have a harmonized method for isolation and identification of *C. jejuni*/*C. coli* within the framework of the AMR monitoring.

The plan is to collect samples and use these for more than one purpose, i.e. for example *Salmonella* and *Campylobacter*. As for the sampling strategies, active monitoring in healthy animals and meat is planned and to have even sampling over the year. There will be a revision of the definition of the epidemiological unit.

As for the randomised samling strategies, no changes are suggested, only fine-tuning.

For *Salmonella* and *E. coli* we plan to include amikacin into panel 1.

The WG considers that the added value of WGS is indisputable, there is a large variation, though, between the state of play at the different MS's and it is too premature to make a change to a WGS approach now.

Summary of the plenary discussion:

It was observed that there is a very large difference between *C. coli* and *C. jejuni* though the reason for this difference is not known.

It was discussed how to make the transition from AST by phenotypic methods into by molecular methods (WGS). There is a handful of NRLs that are already doing WGS for the specific monitoring. We tried to draw some experience from them as well as from the EFSA/EURL-AR confirmatory testing. Moreover, the proposals/suggestions of the working group were mainly connected to practical considerations.

Country presentation: WGS as a surveillance tool in the NRL – the Irish experience (Rosemarie Slowey, NRL Ireland)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

In 2016, WGS was introduced in the AMR NRL aiming to implement WGS as a routine typing tool.

After some initial challenges, we use WGS 1) to validate results of AST testing before inclusion in the EUSR/national one health report, 2) to assess trends and screen for emerging genotypes, 3) to contribute towards enhanced AMR surveillance, and 4) to provide more robust data.

An example of where we applied WGS for the analysis is related an observation of increased prevalence in an ESBL *E. coli* isolate isolated from broilers.

Where WGS is currently most used is for validation of results when we have growth on a carba-plate.

One of our challenges is that we have are doing QC without a bioinformatician. The information we obtain can therefore be overwhelming, and also, we need to keep in mind that just because we do not get hits it does not mean that there are no genes.

Summary of the plenary discussion:

It was mentioned that some NRL's apply Bionumerics to do the QC. It presents N50, number of contigs, length of contigs and other QC information.

Group discussions – plenum follow-up
Approval of the three EURL-AR EQAS reports:

- EURL-AR EQAS *Salmonella*/*Campylobacter*/genotypic characterization 2018 report
  - Report approved without further comments
- EURL-AR EQAS *E. coli*, enterococci, staphylococci 2018 report
  - As the draft report was sent to the network very close to the EURL-AR workshop, the deadline for comments will be another 14 days from now. The EURL-AR will forward an email to the network with this specific information.
    (Subsequently, after 14 days, the report was approved).
- EURL-AR EQAS Matrix 2018 report
  - As the draft report was sent to the network very close to the EURL-AR workshop, the deadline for comments will be another 14 days from now. The EURL-AR will forward an email to the network with this specific information.
    (Subsequently, after 14 days, the report was approved).

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

WHO Guidelines on use of Medically Important Antimicrobials in Food-Producing Animals (Scott McEwen, University of Guelph, Canada)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

WHO published its Global Action Plan and one of the strategic objectives was to optimize the use of antimicrobial medicines in human and animal health. The Guidelines help to achieve this objective. A foundation of the Guidelines is the CIA (critically important antimicrobials) list which was first developed in 2005 and is now its 6th revision.

The purpose of the Guidelines on use of medically important antimicrobials in food-producing animals is to preserve the effectiveness of particularly those antimicrobials that are critically important to human medicine. The Guidelines are evidence-based and contain several recommendations including the overall reduction in the quantities of antimicrobials used in animals and the termination of the use of medically important antimicrobials for growth promotion and disease prophylaxis in healthy animals.

Summary of the plenary discussion:

Comment that there is an expert group defined in Europe – called AMEX – Antimicrobial Expert consultation. This does not consider macrolides in the same category as fluoroquinolones. The use of aminopenicillins is massive in animal health. Deadline to comment against it is 30 April 2019.

AMR surveillance in the US (Pat McDermott, US Food and Drug Administration)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

NARMS was recently reviewed by an external board of experts to help us plan our next strategic direction. The reviewers stated that the program has a unique opportunity to offer more in the long-standing challenge of AMR. In addition, the board stated that the technological advances in genomics and the new data sources have laid the groundwork to add new partnerships and collaborations.

Among the suggestions, it was suggested that NARMS amend sampling and add other commodities and animal raised with antibiotics including seafood, lamb and veal. We also decided to include a screen for carbapenemase production. In seafood, cattle and pigs.

It was suggested to invest in investigating in potential association in actual use and resistance at the user for example by establishing on-farm longitudinal studies. It was suggested to

incorporate genotype and geographical data and to develop visualization tools to display this information.

In the NARMS programme, all *Salmonella, Campylobacter and E.coli* from cattle, pigs, turkeys, chicken, beef, pork, chicken meat and turkey meat, as well as select *Enterococcus,* are sequenced. All *Salmonella*, most *Campylobacter* and all *E. coli* O157:H7 from humans are also characterized with WGS. NARMS has done extensive studies to show that AMR can be predicted with high accuracy from the genome data alone.

Making the complex data from NARMS accessible is a challenge. FDA developed Resistome-Tracker to help end users explore the resistome in an interactive manner. Work is ongoing to improve Resistome Tracker, and to expand it to other pathogen genomes deposited at NCBI. Metagenomic approaches are also under development as tools to monitor the resistomes in samples from animal origin and for testing of surface waters, including irrigation water for crops. Plans are underway to apply metagenomics to pet foods.

Summary of the plenary discussion:

Pat noted that the NARMS budget was around 10.5 mill USD per year – about 200 persons and sequencing costs, but that this number is low when considering all the collateral support from other federal and state agencies. For example, PulseNet at CDC has invested immensely in putting sequencers over the country, and NARMS isolates are testing using this CDC infrastructure.

It was discussed that WGS alone would miss unknown genes without ongoing phenotyping. Pat stressed that we do not see many new genes in *Salmonella* or *Campylobacter* now that we have done WGS on several thousand. The genomic approach is accurate enough that what if we started today from scratch, we could justify WGS as the sole method for foodborne bacteria. It may be relevant to include a phenotypic screening protocol for the most commonly used antimicrobial agents. Perhaps screening by disk diffusion would be enough, which would save a lot of money.

In relation to what was the most difficult in transitioning from AMR using phenotype to using WGS, Pat mentioned that they are still using phenotyping. While he personally believes phenotyping could be dropped or radically scaled back, many think it is too soon to drop MIC testing completely.

There are some issues in relation to sharing data with/from the industry, e.g. in relation to managing the concerns in making all genomes public. Pat mentioned that this is handled differently for different parts of NARMS. While there are State laws restricting what clinical data can be shared and whether WGS data fall under such rules, retail meat information is not under legal restriction. On the food-animal side, NARMS tests caecal samples at slaughter. For these samples we do not receive the slaughterhouse information that goes with the animal sample. There may be fewer restrictions in the US than in Europe.

AMR in seafood (Heather Tate, US FDA)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

A pilot study was performed with the goals to use data for 1) identify organisms appropriate for full-scale surveillance of retail seafood, and their capacity to harbor AMR genes; 2) provide a reference point for resistance phenotypes/genotypes that are being imported through seafood; and, 3) broader scope of sampling to understand the ecology of AMR in foods.

The pilot study was planned for a duration of one year in 8 states. Each state will collect 8 monthly samples of each, i.e. in total 768 of each commodity (fresh and fozen shrimp and skin-on salmon) for the year. This puts us at 80-85% confidence in a 50% prevalence. We estimate that there will be 85%+ of samples from foreign production; 15% domestic. We will narrow down the organisms based on prevalence and AST findings from pilot and plan to begin routine surveillance of AMR organisms in seafood in 2020.

The method for isolation of *Salmonella*, *Vibrio* and other organisms from the samples are still under development.

AMR monitoring in seafood, the ASK project (Valeria Bortolaia, EURL-AR)
See presentation direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx

The ASK project (**A**MR in **S**eafood as common ground for **K**nowledge exchange and risk assessment) is funded by EFSA for 15 months in 2018-2019. The three main objectives of the project are:

- to share knowledge and expertise on antimicrobial resistance (AMR) in seafood.
- to define methodology and guidelines for data collection and AMR monitoring in seafood.
- to identify knowledge gaps for conducting an accurate risk assessment analysis (RA), including the data needed for the identification and characterization of the hazard.

Within these objectives, the five institutions participating in ASK identified different aims for AMR monitoring in seafood, which include:

- to estimate the risk for the consumer
- to assess AMR contamination of the marine environment
- to measure AMR in farmed fish

- to measure AMR in fish pathogens

The possible methods for sampling and for antimicrobial susceptibility testing as well as the bacterial species targeted will vary according to the aim of the monitoring. Furthermore, a simplified classification of seafood that keeps into account the biology of the different seafood species is proposed, with the main aim to simplify the sampling strategy.

The project results will be made publicly available through EFSA channels.

Friday, April 26th 2019

The IMPART project (Kees Veldman (the Netherlands), Sophie Granier (France), Jannice Slettemeås (Norway))

See presentation (direct link (Kees), direct link (Jannice), direct link (Sophie) or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

IMproving Phenotypic Antimicrobial Resistance Testing (IMPART) is a 2018-2019 project and thirteen partners from nine countries are involved. The project consist of 5 different workpackages.

WP1: Selective isolation and detection of colistin-resistant *Enterobacteriaceae* (presented by Sophie Granier)

IMPART looked into plates available for purchase in all EU MS as of September 2018:

- MacConkey + 2mg/L
- ChromAGAR COL-LAPSE
- ChromID Colistin R
- Super polymyxin

After testing we concluded that none of plates worked well – there was poor specificity and we could not get rid of *Proteus*.

Though, now we seem to have a protocol now that works. This protocol is in two steps and is also practical. There is a pre-enrichment for 3 hours. After this there is a selective enrichment with colistin, incubating for 18h-24h. From the incubated broth, we perform a multiplex PCR and check if we have PCR-positive broth.

For the moment, 11 laboratories agreed to participate in the ringtrial in June 2019. Samples will be forwarded together with some selective, ready-to-use media (not commercially available).

WP2: Selective isolation and detection of carbapenemase-producing *Enterobacteriaceae* (Presented by Jannice Slettemeås)

For this WP we already have a protocol. We wanted to find a harmonized method for the selective isolation, detection and characterization of carbapenemase-producing *Enterobacteriaceae* (CPE). Different selective agar plates are used in the laboratories. When you order them, it may take a lot of time until they are available at the laboratories.

The workflow for this WP is similar workflow to WP1, and there will be a final ringtrial in September 2019.

WP3: Establishing new ECOFFs (Presented by Kees Veldman)

The aim was to establish ECOFFs for veterinary antimicrobials, to improve harmonization of AMR monitoring in animal pathogens and to support defining clinical breakpoints of antimicrobials for veterinary use.

Testing will be ongoing in May 2019. Kees will do data-analysis and hopefully soon have new ECOFFs ready.

Three different panels will be used for the testing for the bacteria, starting with very low concentrations. Finally, >50.000 MIC value's will be available as a basis for defining new ECOFFs.

Summary of the plenary discussion:

To establish an ECOFF, the minimum would be to base it on results from five different sources including at least 15 isolates per distribution, i.e. the minimum number of isolates is 75.

When a positive *mcr*-PCR is obtained, the species ID should be looked further into, for example by going back to the broth and perform sequencing.

Survey on the use of NGS across NRLs (Valeria Michelacci, EURL-VTEC)

The aim of the InterEURLs Working Group on Next Generation Sequencing is to promote the use of WGS.

The WG deals with all aspects of NGS and has a long list of deliverables mainly consisting for example in guidelines documents for easy access to available protocols, bioinformatics tools and training modules. It also follows the activity of the ISO working group for NGS standardization.

The NGS survey was conducted in March-April 2018 and the results shoewd that about half of the NRLs already applied sequencing to some extent, i.e the other half does no sequencing. For this reason a follow-up survey was conducted, highlighting that the lack of funding and competence are the main hindrances, but also the lack of perception of the importance of using NGS is still present in some NRLs.

The survey revealed that many NRLs use WGS for research projects, but many already apply WGS during outbreak investigation and in some cases also for monitoring and surveillance purposes. Many of the NRLs declared to be able to perform cluster analysis through whole genome SNPs comparison or wg/cgMLST, but different strategies are still used by using in-house pipelines , commercial softwares or online servers. Many of the participating laboratories have participated in at least one PT on NGS and are interested in participating in future PTs.

A survey dedicated to the use of basic bioinformatics tools was performed reveiling a good agreement in the use of FASTQC , Trimmomatic and SPAdes v3.8 or higher for quality check, trimming and assembly, respectively. On strain characterization, different tools, but shared databases, are used to perform MLST, serotyping and detection of virulence and resistance genes.

The conclusion of the survey is that the majority of the EU MS perform sequencing. Still, many indicate that there are hindrances in the application of NGS, mainly due to the lack of funding. There appears to be agreement on FastQC, Trimmomatic and SPAdes for basic analysis.

Summary of the plenary discussion:

For cluster analysis (cgMLST of wgMLST) it would be useful to harmonize the schemes and pipelines used. Benchmarking can be useful in this respect.

As for the funding issue, this can't be addressed by EURLs and will be eventually solved if WGS will become part of a legislation. However, for the lack of capacity the EURLs will go on working to build preparedness in the use of NGS. The lack of perception, instead, needs to be addressed either trough continuous communication by EURLs or by application of a clear management policy.

Tools for AMR detection (Pimlapas Leekitcharoenphon (Shinny), DTU Food)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

A number of tools to detect AMR genes are available and all have pros/cons: ARG-ANNOT (Antibiotic Resistance Gene-ANNOTation), ARDB (Antibiotic Resistance Gene Database), CARD (The Comprehensive Antibiotic Resistance Database), Resfams, SSTAR (Sequence Search Tool for Antimicrobial Resistance), ResFinder.

ResFinder deals with both assembly based and read based sequences and has both a webbased and a standalone commandline version.

Machine learning and deep learning will be relevant to future tools to detect AMR genes.

Summary of the plenary discussion:

For the tools run in commandline version, as long as you work with assembled genomes, you can use your local computer. But if you would like to work with more than 20-30 genomes in raw reads at the same time, you will need to have access to a server.

The EFFORT-project – lessons learned (Patrick Munk, DTU Food)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

Previous to EFFORT (Ecology from Farm to Fork Of microbial drug Resistance and Transmission), we were surprised how well WGS reflected the use of antimicrobials at the farm.

As part of the EFFORT project, samples from pig and poultry production were taken all over Europe. Faecal samples were pooled and DNA was extracted directly from this. Not all samples could be sequenced using the method we wanted to apply, though, we found that sequences from pigs were quite different from sequences from poultry.

As for the total AMR load there is quite a lot of country difference and much difference within each country.

Next step is to look into the sequence fragments and try to get 100 genomes out of them.

Summary of the plenary discussion:

This type of technique would be applicable for monitoring. It gives much information as to the epidemiology. It would be rewarding to have this be part of routine monitoring worldwide. Currently it requires computerpower and skilled staff, though. For the future, cloud compute services could be relevant so that data can be uploaded to a hub. Moreover, the tools can be automated. It is costly, but we should keep in mind that we need to look at this with one health approach – this technique is helpful not only for AMR monitoring. Currently the software can take one metagenomics sequence at a time.

Method for AMR surveillance (phenotypic/genotypic) (Kees Veldman, the Netherlands)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

With the aim to prepare for using WGS in AMR monitoring we have looked into using phenotypic and genotypic methods for AMR surveillance.

Currently, all European countries deliver the same type of AMR-data for monitoring purposes. However, there are disadvantages of the phenotypic AMR: the number of antimicrobials we test is limiting. Using phenotypic AST and WGS we compared 150 well described *E. coli* from broilers, slaughter pigs and veal calves (EFFORT project). The results reveal differences in the concordance depending on the antimicrobial class: e.g. there is a high diversity in aminoglycoside genes and solely testing gentamicin is not a good indicator of all aminoglycoside resistance because many different resistance genes are involved. For sulphonamides there appears to be a difference in the MIC-values obtained between the animal

species (probably due to reading of the endpoints (re-testing of MIC's have not been performed)). For most other antimicrobials like tetracyclines the concordance between phenotypic and genotypic testing is really high.

In general, phenotype (MICs) versus WGS proved to provide very similar results. WGS provides additional information, though, about the epidemiology of AMR: plasmid types, ST types, etc. To make the transition to do routine monitoring by WGS, we need to optimize our pipelines to make it suitable on a larger scale and to reduce the costs.

Phenotypic and genotypic detection of colistin resistance (Ana Rita Rebelo, DTU Food)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

It is a work in progress to have and keep an overview of all the genetic mechanisms of resistance, including *mcr*-genes and their variants.

Using bioinformatics tools for prediction of resistance implies an understanding of their thresholds. Furthermore, a negative genotypic test cannot be used to predict susceptibility.

Broth microdilution remains the only recommended phenotypic method for detection of colistin resistance. Evaluation of commercial systems based on broth microdilution is available on EUCAST website (www.eucast.org).

In general, for quality assurance, both susceptible and resistant strains should be used.

Summary of the plenary discussion:

Note that the WHO report ('The detection and reporting of colistin resistance') describes the handling from when you already have the strain. It does not describe the detection of an *mcr*-positive strain in a sample.

Measures taken in the NL to reduce the use of antibiotics in animal husbandry – monitoring results and the effect of reduced antibiotic use on resistance data (Michel Rapallini, the Netherlands)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

Within the framework of the Dutch national residue monitoring plan, microbial screening for antibiotics has been performed. A Nouws Antibiotic Test (NAT) screening has been done by applying five paper disks impregnated with renal pelvis fluid from kidney into punch holes in five test plates and supplemented with buffer. After overnight incubation, inhibition zones can be registered. Further analysis showed that there was a nice correlation between the inhibition zone (kidney fluid) and the residue concentration in meat.

Antibiotics in meat you can only see after slaughter, therefore screening of antimicrobials in the chicken feathers was also performed as the feathers indicate that there was antibiotics use, for example presence of antimicrobials in the tip of the feather would indicate antimicrobial usage in the early life of the bird. Moreover, for this, on-site dipstick sampling is possible. Can be done with hairs too.

The sale of antimicrobial for veterinary use has decreased drastically. One of the reasons is that antimicrobials may now only be prescribed by veterinarians and only sick animals can get antimicrobials.

Goal: In the end we can knock out the resistant bacteria!

Summary of the plenary discussion:

It was discussed if the decrease in use of antimicrobials of circa 50% had influence on animal health and welfare. Michel stated that to his knowledge this was not the case.

As for flock medication, if the whole flock is sick, yes, you can treat the flock.

The feather method appears to be very robust. We had feathers that were stored for several months in the freezer.

Legislation is not the only way to reduce antimicrobial consumption. We need to lower the need for antimicrobials. This is the goal. Legislation could probably help start thinking in this direction but will not get you the whole way.

In fact, in NL, we used a lot of preventive medicine. Also, we need to consider that farming is a business. Lots of collaboration between the farmer and the veterinarian has proven efficient.

Activities at the NRL-Norway, including eradication of MRSA in pigs (Jannice Slettemeås, Norway)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

As of June 2019, AMR will be notifiable in Norway. LA-MRSA, fluoroquinolones resistant, ESBL-, AmpC-, and carbapenemase-producing bacteria will be included as notifiable.

For Livestock Associated-MRSA (LA-MRSA) in swine, we looked for MRSA through NORM-VET from 2008 to 2012. In 2014, we got a separate surveillance system for LA-MRSA in pigs following our national action plan where it is stated that LA-MRSA should not be established in the Norwegian pig population. Since 2014, we follow the search and destroy strategy, which is successful due to the Norwegian geography,

Work is ongoing to introduce WGS and bioinformatics through an internal strategic project called SEQ-TECH. Currently we are considering a number of questions in relation to the infrastructure and handling of the samples/data – in-house/outsource? Where to store data? Where to perform the bioinformatics analysis?

Summary of the plenary discussion:

In Norway we found some MRSA in cattle, also. We checked cattle and pigs at the same farms. We retest during the year two or three times.

In other countries the level of MRSA is currently too high and it is now much too late to start an eradication programme.

MRSA prevalence in Swiss slaughter pigs since 2009 (Gudrun Overesch, Switzerland)
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

In Switzerland we started monitoring for MRSA in 2009 and were happy to not find anything in poultry and only little (2%) in pigs. We looked at nasal swabs from slaughterhouses. As for fresh meat, some MRSA was detected in foreign chicken meat analyzed in 2014.

We then detected increased MRSA in Swiss slaughter pigs: the *spa*-type t34 was the most prevalent one. It was detected geographically over Switzerland, from the beginning there seemed not to be a common source. In 2012 – no region seemed to be crowded with positive herds.

A case control study was done in 2013 and the analysis did now show a common source or risk factor indicating MRSA transmission. Followed up with a longitudinal study testing sows and offspring. All the way through fattening period and to slaughter. The negative farm was also astonishing: We found that the slaughter house is a very important parameter. The negative animals were found positive when sampled (nasal swab) at the slaughter house.

In 2018 the MRSA prevalence in pigs rised to 44%. Most of the fattening pigs – these are almost all LA-MRSA, but nowadays spa type t011 is responsible for the ongoing increase. With WGS we are now looking into finding out why *spa*-type 11 is so spread and why it is so different from *spa*-type 34.

Summary of the plenary discussion:

It was commented that for example in Denmark there are MRSA screening programmes at hospitals if you are admitted and work with pigs/mink. This is also recommended in Switzerland. Though, Gudrun mention that most of the hospital personnel she has contact with now really trust their hygiene procedures and therefor no longer take special measures for screening.

Sampling at farms or slaughterhouse was discussed. If sampling at the slaughterhouse, you may overestimate the number of positive farms. There may, though, be differences between slaughterhouses and how they hold the animals. Gudrun mentioned that they will propose hygiene measures also at the slaughterhouses. For example to have a resting time for 2-3 hours before they go into slaughter. They tested the keeping ground before the pigs arrived and it was positive.

It was mentioned as an eye-opener, that in the case of *Salmonella*, a *Salmonella* expert in an MS where *Salmonella* is very prevalent said that this in fact was related to one distributor.

Homemade bead array dedicated to the monitoring of the spread of critical antimicrobial resistance determinants in Gram-negative bacteria (Cécile Boland, Belgium)

The homemade bead array is less expensive in consumables than WGS and commercial arrays and than performing multiple PCRs and for analysis of the results, only excel is required. Moreover, it is fast and cheaper than WGS.

We also have an array for gram positive upcoming and a research project on linezolid resistance. In this context, if any have linezolid-resistant MRSA or enterococci, please be in touch. We would like to test them also.

Summary of the plenary discussion:

The reagents costs are about 20 EUR per strain and one of the big advantages is that it is easy to add genes. There is a limit to the number of probes. Currently there are 41, and 80 different targets are possible to multiplex at once. The length of the arms are 40 nucleotides (20-30 per arm) and it is sensitive at the SNP level.

General discussion of accreditation of sequencing for AST – what are the opinions on this and the approaches at the laboratories?
See presentation (direct link or https://www.eurl-ar.eu/presentations/workshop-kgs-lyngby-april-2019.aspx)

Agenda item omitted due to time constraints.

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 2020, we aim at arranging a joint meeting with the FWD-network.

Appendix 1
Summary from group discussions at the EURL-AR workshop 2019

Breakout groups at EURL-AR Workshop 2019

Dear EURL-AR workshop participants,

At the EURL-AR workshop 2019, we will have time to go into break-out groups to discuss the experiences and challenges encountered following the performance of broth microdilution.

Discussion items have been drafted for the groups to consider. After the groups have discussed internally, Rene Hendriksen (EURL-AR) will moderate a plenum summary and discussions.

In order to obtain the best input for the group discussions, all NRL participants are 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.

All workshop participants are invited to also bring up any important observations with regard to the 2018 EQAS's. This could be challenges faced or other observations made during the testing, or it could be remarks to the draft reports.

Best regards,

EURL-AR

For discussion:

1) When you test *Campylobacter* to identify whether they are *C. coli* or *C. jejuni*, which method and protocol do you use? And have you encountered problems with the method?

   Comments at the plenum follow-up session:

   MALDI-TOF appears to be widely used, as well as PCR (different protocols). One laboratory used phenotypic characterization for the purpose, and a negative hippurate test would lead to testing the isolate using PCR (the risk of false positive hippurate results was mentioned).
   Based on the results from the EURL-AR EQAS, it would appear that till now, these methods have not caused issues.
   Problematic strains have been identified during projects, and for these, the problem was solved by WGS and analysis of the obtained sequences.
   Potentially harmonization of PCR protocols to distinguish between *C. coli* and *C. jejuni* would be relevant. This would, however, be an issue to discuss with the EURL *Campylobacter*.

2) What measures do you take to ensure that strains are not switched during the testing procedures?

   Comments at the plenum follow-up session:

In the routine work, tests are generally not repeated, and testing and switches might not be discovered. But in the EQAS's and in the confirmatory testing, switches are discovered. In general, as strain management procedures has been introduced to prevent switching of strains, the network did not find that this was an issue. QC-procedures included ensuring that when unpacking strains, they are streaked one isolate on one plate, i.e. one strain per plate, and registered in LIMS labelled with the LIMS number. Moreover, a general suggestion was to limit the number of strains the technician is actively working with. Though, in spite of good QC-procedures, human mistakes happen, for example when an 'un-experienced' technician is helping out. Even if a team is extremely experienced, it is vulnerable.

3) How do you read the MIC if the growth in a test-range ends up with a micro pellet in the last well with visible growth – do you ignore the micro pellet or do you strictly encounter all visible growth as growth?
Do you always read by the same approach, or do you take into account any previous knowledge on prevalence of AMR profiles when reading MIC values?

Comments at the plenum follow-up session:

There are different approaches to reading an endpoint depending on the antimicrobial and organism. If a pellet is less than a dot, it was, however, generally ignored. Also, it would be taken into consideration if the potential endpoint was above the breakpoint or not. Others mentioned that micropellets are counted as growth and if it leads to an unusual profile, they would look further into it.

4) When you receive bacterial cultures for an EQAS, do you handle them differently compared to routine testing (e.g. perform additional tests) – and what is the reason for that?

Comments at the plenum follow-up session:

In general, routine procedures were not followed when receiving EQAS test strains. It was mentioned that good care is taken during the testing of EQAS test strains, for example by performing repeated testing by different people. One laboratory, though, specifically mentioned that they do not treat the EQAS test strains differently and also mentioned that they generally do not do much retesting as they would expect different phenotypes when performing an EQAS.
The EURL-AR will look into phrasing clear guidelines on this matter in the EQAS protocol.

5) How do you ensure that your laboratory obtains pure strains when isolating bacteria from samples?
How do you ensure that you perform AST on pure cultures when you observe a contaminated culture on a plate?

Comments at the plenum follow-up session:

It was mentioned to ensure to culture from a single colony and try to have dispersed growth. Blood agar was most commonly used media. Non-selective chromogenic agar are also used now – designed for urine analysis and are a bit more expensive than blood agar but is a good pedagogical tool.
It was mentioned that the most dominant morphotypes from the plates are picked. For *Salmonella*, if observing a very exotic serotype or a serotype belonging to an unexpected origin, it would be purified and serotyped again.
Contaminations with *Proteus* are an issue. *Proteus* are observed under *E. coli* and *Staphylococcus* colonies.

6) How do you deal with the discrepancies between the method guidelines given by CLSI, ISO 20776:2006 and Trek Diagnostics?
(*See supplementary information in the table below*)

Comments at the plenum follow-up session:

The breakout groups realized that the applied method had many discrepancies between the NRL's. Both CLSI, TREK and ISO 20776 are used. All laboratories use strongly validated methods and the question is to which level should a method be harmonized – should each step be harmonized? Currently, we focus at the result – that it is reproducible and reliable.
It was mentioned, however, that even if a laboratory refers to a specific guideline/standard as the reference, it may in fact not be followed in all details.
In general, there appeared to be pros/cons for the different guidelines/standards, and it was suggested to include the method details in the analysis of deviations observed in an EQAS.Also, it was suggested to start the analysis by looking at the outcomes of the QC strains, e.g. the *Enterococcus* strain.
From the EURL-AR we will take the initiative to contact TREK to ask them for evidence that their method is comparable to CLSI.

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Bacteria	Guideline	Solvent for preparing MacFarland 0.5 suspension	Inoculum size*	Incubation temperature	Incubation time	Methods for colony counts
E. coli and *Salmonella*	ISO 20776-1:2006	MH broth or saline	5×10^5 CFU/ml (range $2 - 8 \times 10^5$)	34 – 37 °C	16 – 20 h	A
	TREK	Water	1×10^5 CFU/ml (range $5 \times 10^4 - 5 \times 10^5$)	34 – 36 °C	18 h	B
Enterococcus and *Staphylococcus*	ISO 20776-1:2006	MH broth or saline	5×10^5 CFU/ml (range $2 - 8 \times 10^5$)	34 – 37 °C	16 – 20 h	A
	TREK	Water	1×10^5 CFU/ml (range $5 \times 10^4 - 5 \times 10^5$)	34 – 36 °C	18 – 24 h	B
Campylobacter sp.	CLSI	MH broth or saline	5×10^5 CFU/ml (range $2 - 8 \times 10^5$)	36 – 37 °C	48 h	C
				42 °C	24 h	
	TREK	MH broth	10^6 CFU/ml	35 – 37 °C	48 h	B
				42 °C	24 h	

**) Transfer of 50 µl MacFarland 0.5 suspension to 10 ml of MH broth equals an inoculum size of 5×10^5 CFU/ml. Transfer of 10 µl equals 1×10^5 CFU/ml.*

METHODS FOR COLONY COUNTS:

A. ISO: Transfer 10 µl from control well to 10 ml of broth/saline -> streak 100 µl to agar -> incubate -> 20-80 colonies to be expected.

B. TREK: No current recommendation. Latest available method (Trek doc., 2012): Streak 1 µl from control well to agar 1. Transfer 1 µl from control well to 50 µl of water and streak 1 µl to agar 2. Incubate agar 1 and 2 --> read plates. Look up the inoculum range in a table (not presented here) by combinating the two counts.

C. CLSI: For *E. coli* ATCC 25922, transfer 10 µl from control well to 10 ml of saline -> streak 100 µl to agar -> incubate -> ~50 colonies to be expected.