Foreword

The WG has been established by the European Commission with the aim to promote the use of NGS across the EURLs' networks, build NGS capacity within the EU and ensure liaison with the work of the EURLs and the work of EFSA and ECDC on the NGS mandate sent by the Commission. The WG includes all the EURLs operating in the field of the microbiological contamination of food and feed and this document represents a deliverable of the WG and is meant to be diffused to all the respective networks of NRLs.

Overview of conducted and planned Proficiency Tests

Introduction

Next generation sequencing (NGS) is a new emerging technique in food microbiology, which currently is becoming increasingly mainstream since it provides fast results for an affordable price. Whole genome sequencing (WGS) of important microbes can easily be carried out and the results contain more background information compared to traditional phenotyping or other molecular techniques such as serotyping, pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST), where only a small part of the genome is analysed. Compared to traditional typing methods, WGS and genotyping offer significant advantages since it is possible to carry out analyses regarding antibiotic resistance, virulence, serotyping, MLST and phylogenetic relationship using one single technique. In addition, WGS data analysed for single nucleotide polymorphisms (SNPs) provide a much higher resolution compared to e.g. pulse-field gel electrophoresis. Therefore, NGS is an excellent tool for surveying antimicrobial resistance, virulence and outbreaks of various important food pathogens.

Objectives

The objectives for this report is to investigate ongoing and planed NGS based Proficiency Tests (PTs) tendered for NRLs in EU and to identify synergies and deficiencies in previously delivered PTs.

Methods

A survey was performed in the autumn of 2019 among all EURLs of the joint WG EURLs NGS, to collect detailed information about conducted and planned PTs on NGS and to exchange experiences of PTs performed. In addition, the EURLs have in 2020 we reported their status regarding the progress of getting NGS and WGS implemented in their laboratories.

Results

The present document comprises of a summary of the feedback on the following topics and finally a conclusion:

1) Previously conducted NGS-based PTs.
2) Planned NGS-based PTs for the EURL work programs 2020-2022.
3) Lessons learned in relation to NGS-based PTs.

In the following, each of the eight EURLs will be named in short as SAL (EURL-*Salmonella*), CAM (EURL-*Campylobacter*), LIS (EURL-*Listeria* monocytogenes), CPS (EURL-coagulase positive staphylococci), VTEC (EURL-VTEC), VIR (EURL-Foodborne viruses), PAR (EURL-Parasites) and AR (EURL-Antimicrobial resistance).

Previously conducted NGS-based PTs.

Five of the EURLs have conducted and provided NGS-based PTs in previous years:

At CAMP, a voluntary pilot-PT "Subtyping of *Campylobacter jejuni*" was provided in 2019 to the NRLs for *Campylobacter*. The PT-25 included eight samples of pre-extracted DNA to be sequenced by any method preferred. Participants were requested to report MLST-types, and if WGS was performed participants were asked to do cluster analysis. If participants performed WGS they were asked to submit raw data, assemblies and cluster images used for interpretation. The aim was to identify differences in methods and the extent of deviations in results. Performance thresholds were not defined, but the MLST results were scored (one point for each correct defined ST). The cluster analysis was optional and was not scored. The WGS data was evaluated in relation to selected QC parameters (not included in the report but presented at the EURL-workshop 2019). In total, 21 laboratories participated in the PT, and 16 in the part on cluster analysis. The scores for determining STs were overall very good, with better results for laboratories performing WGS than Sanger sequencing. The interpretations made from the cluster analysis were to a high extent concordant.

At LIS, two PTs were conducted for the NRLs on *Listeria* typing. In 2017, the PT for *Listeria monocytogenes* typing was based on molecular serotyping, PFGE, and MLST and in 2018 the *L. monocytogenes* PT included cluster determination in addition to previously mentioned methods. The different PT included 10 bacterial cultures of *Listeria* and participants were requested to use the preferred in-house procedure of DNA extraction and NGS, followed by submitting raw data (fastq-files), matrix distance and the phylogenetic tree. LIS performed all further analysis by use of an in-house developed pipeline and with the commercial software's for QC, trimming, assembly, assembly statistics, MLST, serotyping, determination of the clonal complex and finally, the cluster analysis. The aim was to evaluate the competences of the NRL network in WGS analysis and the quality parameters of the sequences and their effect on characterization of *L. monocytogenes* by WGS, but also to evaluate the interlaboratory- and platform variability in terms of SNPs and allelic differences.

At SAL, the annual PT on *Salmonella* serotyping provided to the NRLs for *Salmonella* in November 2019 was expanded to include 10 extra isolates for voluntary participation on cluster analysis with free choice of molecular methods (or combination of methods). A total of 18 NRLs participated in the cluster analysis, with 6 participants for PFGE analysis, 8 for MLVA analysis and 14 participants for WGS analysis. Evaluation (per methodology) of the participants' cluster analysis results was done on the ability to correctly identify cluster(s) of genetically closely related isolates, as pre-defined by the EURL-*Salmonella*. However, cluster definitions

may vary depending on the situation or the specific research question, e.g. in outbreak investigations or surveillance. The participants of this pilot PT were free to use their own interpretation of "cluster(s) of closely related isolates". Therefore, no performance criteria were set for this pilot PT on cluster analysis. As a minimum, it was expected that the participants would report the technical duplicate strains SCA03 and SCA06 to be (part of) one cluster which was indeed reported by 12/14 participants. Unfortunately, these duplicate strains showed more variety in their WGS data than expected. This was observed during the PT in November 2019, as well as after storage (at room temperature) of the transport tubes containing the strains until February 2020 (tested at the EURL). Further investigation to explain this variety is still ongoing (November 2020).

At VTEC, one PT was conducted (2017) for the NRLs for VTEC and for the Italian Official Laboratories. The PT included six bacterial cultures of STEC and participants were requested to use the preferred in-house procedure of DNA extraction and NGS, followed by submitting raw data (fastq-files). The VTEC performed all further analysis by use of an in-house developed pipeline for QC, trimming, assembly, assembly statistics, MLST, serotyping and virulotyping - and finally, VTEC performed a phylogenetic analysis. The aim was to evaluate the quality parameters of the sequences and their effect on characterization of STEC by WGS, but also to evaluate the interlaboratory- and platform variability in terms of SNPs and allelic differences. Later on, in novel PT rounds the possibility to characterize with WGS pathogenic *E. coli* strains shipped as soft agar cultures was included as an alternative to classical methods, by requesting only the characterization results and no intermediate output file (e.g. fastq files). In detail, this approach has already been used in PT23 (2018) and PT26 (2019), focusing on the detection of the main STEC virulence genes (*eae* and *stx* genes), the identification of a range of relevant STEC serogroups (at least the top-13 serogroups) and the subtyping of Shiga Toxins (Stx)-coding genes. The PTs also included a facultative exercise on cluster analysis aimed at the identification of clusters of isolates based on genomic analysis based on PFGE in 2018 and either on PFGE or WGS (SNPs analysis or cgMLST) in 2019.

At AR, GMI (Global Microbial Identifier) PTs in 2015, 2016 and 2017 have been conducted by the EURL-AR. The PT's were aimed at a global audience based on the GMI community. Moreover, when the PT was originally initiated, invitations to sign up were forwarded to the relevant EURL-AR international contact lists. The GMI PT consisted of two components and of which the EURL-AR was in charge of the wet-lab component. The target audience for the wet-lab component was laboratories performing wet lab sequencing including library prep. For each iteration of the GMI PT, the material provided to the participating laboratories was related to three bacterial organisms (two of each organism). For each of the six bacterial organisms, both a pure culture of the live organism and pre-prepared extracted DNA was forwarded as

test material. The objectives for the wet lab component of the GMI PT was for the participating laboratories to perform DNA extraction, purification, library-preparation, and WGS of the six bacterial cultures for each iteration. Moreover, to whole-genome-sequence the pre-prepared DNA of the same six bacterial strains that were sent as cultures. Participants were requested to upload reads to an ftp-site and optionally also identify the MLST of the strains as well as the resistance genes present in the strains if that is something that is routinely done within the laboratory. As of 2017, the submitted reads were analyzed by the organizers and were evaluated in relation to a number of quality metrics. Based on the scores obtained for the quality parameters, an overall score was reported to the individual participant. The wet lab GMI PT system to analyze the quality metrics was a Pearl / R- script developed by DTU Food hosting the EURL. Unfortunately, the analysis itself was manually conducted and "bugs" in the algorithms and coding made it very difficult to provide timely results to the participants. This affected the iterations with too little time to provide summary reports. In addition, the mode to capture and compare the reported predicted MLSTs and resistance genes present was similarly not sufficient and effective as being conducted by using a survey mode. The use of the survey resulted in a huge workload and manual hand-hold analysis. Thus, the non-funded GMI PT was terminated in 2018 due to the continued changes and updates of the coding and the work intensive analysis.

Planned NGS-based PTs for the EURL work programs 2020-2022

In general, five of the eight EURLs have scheduled to continue carrying out NGS-based PTs in the years to come.

At CAM, a voluntary pilot-PT "WGS of *Campylobacter*" was provided in 2020 to 19 NRLs for *Campylobacter*. The test consisted of extracted DNA from two different *Campylobacter* strains and lyophilised bacteria of corresponding strains. The aim of this PT-28 was to quantify differences between WGS data from *Campylobacter*, produced at different laboratories from DNA prepared at the laboratory or from DNA delivered by the EURL. Participants were requested to submit raw sequence data and the data was evaluated based on different QC parameters. The progress of the report for PT-28 is ongoing. In 2022, a voluntary PT for WGS-based subtyping mimicking an outbreak investigation, will be performed with a similar outline to PT-25 organised in 2019, but using strains instead of DNA.

At LIS, a PT test on *L. monocytogenes* typing was in 2020 based on WGS and the participants received a two-part analysis consisting of a wetlab and drylab part with 10 isolates and 6

fastq for drylab part. When submitting results, the participants is requested to inform about the method applied (SNP, Cg-wgMLST) and all the parameters used for QC.

At SAL, also in 2020/2021 the annual PT on *Salmonella* serotyping provided to the NRLs for *Salmonella* will include extra isolates for voluntary participation in the cluster analysis part with free choice of method (PFGE, MLVA, WGS). The set-up of the PT will be similar to that of 2019, with the difference that the PT of 2020 mimicked an outbreak situation more clearly since it included the disclosure of the MLVA type and WGS data of a reference strain.

At VTEC, the annual PT on identification and typing of STEC and other types of *E. coli* will be provided to the NRLs for *E. coli* for 2020 and 2021. For the typing component, the method will be free of choice as already done in 2018 and 2019. If WGS is performed, the results can be reported instead of results by conventional methods. The participating laboratories will be requested to indicate the typing method used (e.g. PCR or WGS). Performance will be scored based on the ability to characterize the strains correctly, regardless the method used. Cluster analysis will be left as facultative and, differently from previous PT rounds, the accepted methods for this part will only be WGS-based, including either SNPs analysis or cgMLST analysis, but excluding PFGE.

At AR, In the EURL AR work plan for 2020, the past GMI PT was included with the intention to perform an overhaul of the past coding to update and finalize these as well as to construct an informatics IT module to report MLST and AMR genes and chromosomal point mutations. The new PT names “DTU Genomic EQA” will be offered to the National Reference Laboratories (NRLs) of the EURL-AR network performing WGS. The objectives of DTU Genomic PT is assess the quality of the WGS and the ability to detect AMR determinants and predict phenotypic resistance of the participating NRLs. This will allow the EURL to assess the performance of the NRLs based on a set of WGS quality metrics and their analytic capacity to identify AMR determinants and predict phenotypic antimicrobial susceptibility profile in the test cultures based on the obtained sequence. For the DTU Genomic PT, the material provided to the participating NRLs will be related to bacterial organisms relevant to the EURL-AR monitoring program i.e. *Salmonella enterica*, *Campylobacter jejuni/ coli* and *E. coli*. For each of the included bacterial organisms, both a pure culture of the live organism and pre-prepared extracted DNA will forwarded as test material. The NRLs should perform DNA extraction, purification, library-preparation, and WGS of the bacterial cultures. Moreover, to whole-genome-sequence the pre-prepared DNA of the same bacterial strains sent as cultures. Participants will be requested to submit reads to a ftp site and submit the identified resistance genes present in the strains to an informatic IT module. The submitted reads will be evaluated in relation to a number of quality metrics and identified AMR determinants will be analyzed by the EURL-AR. Based on the scores obtained for the quality

metrics and detected AMR determinants, an overall score will reported to the individual The NRLs.

As a final comment on this topic, the CPS, VIR and PAR inform, that they have no plans for PTs in the coming years due to lack of current NGS activities at almost all the NRLs in their network.

Lessons learned in relation to NGS-based PTs.

At CAMP:

- The inclusion of a scenario to make the PT resemble an outbreak investigation was appreciated.
- There were surprisingly small differences between data (both assembly and raw data) generated at different laboratories with different sequencing techniques and modified with different software/pipelines.
- The variation in DNA concentration as measured by participants was very high.

At SAL:

- None of the participants reported the cluster identification completely as expected and as defined by the EURL-*Salmonella* interpretation in the PT of 2019. In this study, participants were free to use their own definition of cluster analysis and ‘closely related strains’, which may have resulted in differences in cluster identification with WGS-derived data.
- Two technical duplicate strains showed more variety in their WGS data than expected (PT 2019). Further investigation to explain this outcome is still ongoing (November 2020). The results of the first pilot PT on cluster analysis (including NGS) have been evaluated, concluding that more guidance on the research question was needed. For this reason, the next PT on cluster analysis (November 2020) included the information that the PT would mimic an outbreak situation of a specific *Salmonella* serovar, including MLVA type and WGS data of the reference strain.

At VTEC:

The high participation rate in the first PT dedicated to NGS (2017) confirmed the interest in this emerging technology. Participants performed WGS as routine and performed correct characterizations even though no sequencing specifications were provided. The sequences produced were highly heterogeneous in between laboratories, but also within the same laboratory, indicating non-standardized in-lab workflows. SNP analysis was always effective in detecting the correct clusters, cgMLST provided a good resolution of the clusters, while wgMLST did not (see appendix for more details).

European Union Reference Laboratory Foodborne Viruses

- About half of the NRLs chose to use WGS when this alternative possibility was given (2018 and 2019) and the wide majority performed well in the characterization of test strains, providing more detailed typing of the strains than those using classical methods (e.g. performed H-typing and identification of additional virulence genes). When performing cluster analysis, more than 70% of the participants chose to use only WGS-based strategies. In detail, cgMLST was applied by the vast majority of them, who could all correctly identify the cluster of isolates, with only 2 exceptions out of 14 labs, one of which consisted in misunderstanding of the question. On the other hand, SNPs analysis was wrongly interpreted for cluster identification by 3 out of 8 participants applying such strategy.

- After the first edition of PT specifically dedicated to NGS analysis in 2017, since 2018 EURL VTEC has adopted the alternative strategy of accepting NGS-derived results without asking for the sequencing files produced, giving the participants a direct feedback on results interpretation and avoiding the collection and reanalysis of such big data, with very satisfying results in terms of participation and results interpretation.

At AR:

- Capturing large files containing reads together with the corresponding metadata from various countries around the world was challenging due to issues related to network connection, software and also other undefined issues
- If not considering very carefully the method for submission of reads, metadata and detected antimicrobial resistance genes, a large amount of manual data handling may be expected (time consuming)
- The complexity of the analyses were higher than expected, i.e. to extract the necessary data for analysis and present this large amount of data and information in a fairly simply manner

In conclusion

NGS is an important emerging technique regarding bacterial typing and outbreak investigation in food microbiology and concordantly, the majority (5/8) of the EURLs are currently providing PTs involving NGS for the NRLs. In this regard, it is considered important to exchange plans and experiences between the EURLs. However, the CPS, VIR and PAR do not provide PTs based on NGS for their NRLs and have no plans of implementing NGS in the PTs the next years. All PTs involving NGS included quality assessment of the sequence data and phylogenetic cluster analysis (except EURL AR that did not included cluster analysis) and the majority had a genotyping approach included as well.

It is expected that the number of participants on NGS PTs - or NGS components of PTs - in general will increase, as more laboratories are expected to implement the method and as the interest for participating on NGS PTs has already shown to be present among several of the networks. Due to the expected interest and the fact that a considerable number of the NRLs are represented in more than one EURL network, the EURLs should, on a common base, aim to limit the annual number of NGS-PTs and identify synergies spanning more than one EURL network and also avoid overlapping of testing periods and deadlines for NGS-PTs of different networks. In addition, various reference strains could be shared between EURLs thereby, obtaining a pool of commonly used reference strains that could be applied for PTs/EQAs within the EURL network.

The working group agreed that delivering PTs for which NGS is accepted as typing strategy in parallel with standard methods is advisable, either for reducing the costs of the PTs or for gathering data useful for validation of NGS-based methods.

Some of the EURLs have conducted specific PTs on NGS and gained experience in the set-up, evaluation procedures and methods for submission and analysis of reads and data. Especially submission of sequences and data – but also the analysis for evaluation - is of concern in order to avoid unnecessary workloads for both the provider and the participants.

At present (November 2020), a new ISO standard regarding guidelines for genotyping of foodborne bacteria (ISO 23418, Microbiology of the food chain — Whole genome sequencing for typing and genomic characterization of foodborne bacteria — General requirements and guidance), is being prepared and the EURL group expects that this ISO standard will be important for future implementation of NGS in the PTs. Additionally, the experiences earned by the EURLs will also be useful for both inspiration and consideration when planning future PTs for the NGS area.